Photoregulation of Enzyme Induction and Cell Differentiation in Tomato Photomorphogenic Mutants

A Thesis Submitted for The Degree of
Doctor of Philosophy

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Certificate

This is to certify that the thesis entitled **Photoregulation of enzyme induction and cell differentiation in tomato photomorphogenic mutants** is based on the results of the work done by **Mr K Venkateshwar Goud** 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.

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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

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TO MY PARENTS

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List of abbreviations

A = absorbance
au = *aurea*
au,hp = double mutant *of aurea* and *high-pigment* mutants
BL = blue light
blu = *blue light uninhibited*
BSA = bovine serum albumin
b = breadth
cab = chlorophyll a/b binding protein
cop = *constitutive photomorphogenesis*
Cot = cotyledons
cue = *cab under expression*
d = day
det = *de-etiolation*
D = dark
DNS = dinitrosalicyclic acid
doc = *dark over expression of cab*
DW = distilled water
EDTA = ethylene diamine tetraacetic acid
ein = *elongated internode*
EODFR = end-of-day far-red
ft^{1}*}'* = *far-red hypocotyl*
fre = *far-red elongated*
fri = *far-red insensitive*
FRL = far-red light
FR = far-red
FW = fresh weight
GA = gibberellic acid
GL = green light
h = hour
hp = *high-pigment*
hy = *elongated hypocotyl*
Hyp = hypocotyl
IAA = Indoleacetic acid
kD = kilodalton
l = length
lip = *light independent photomorphogenesis*
λ_{max} = maximum wavelength
lz = *lazy*

min	= minutes
MW	= molecular weight
NPH	= *non-phototropic hypocotyl*
NF	= norflurazon
NiR	= nitrite reductase
NR	= nitrate reductase
nkat	= nanokatal
pkat	= picokatal
PAGE	= polyacrylamide gel electrophoresis
PAL	= phenylalanine ammonia lyase
pew	= *partially etiolated in white light*
Pfr	= far-red light absorbing form of phytochrome
Phy	= phytochrome holoprotein
PHY	= phytochrome apoprotein
PHY	= phytochrome gene
phy	= phytochrome gene mutant
Pr	= red light absorbing form of phytochrome
pro	= *procera*
R	= red
RL	= red light
SDS	= sodium dodecyl sulphate
TCA	= trichloroacetic acid
TBST	= tris-buffered saline + tween 20
TEMED	= N,N,N',N'-tetramethyl ethylene diamine
TRIS	= (tris[hydroxymethyl]aminomethane)
tri	= *temporarily red light insensitive*
UV	= ultra violet
v/v	= volume/volume
WL	= white light
WT	= wild-type
w/v	= weight/volume
yg	= *yellow-green*

CONTENTS

1. INTRODUCTION

An inherent and essential feature distinguishing living organisms from inanimate objects is growth and development. While growth is innately programmed with the necessary blueprint encoded in the genome, the expression of specific developmental programs such as flowering in higher plants is subject to environmental cues. In fact, such environmental regulation distinguishes plant development from animal development. Moreover, the environmental regulation of metabolism and development allows plants to adapt to a changing ambience and survive under unfavorable conditions. Among the several environmental factors, light plays a very important role in the plant life cycle.

Higher plants perceive light signals in a variety of forms such as directional stimuli leading to phototropic movements of plants; changes in duration of day length, which allow plants to anticipate change of seasons by a phenomenon known as photoperiodism; solar tracking of leaves and organs; detection of shading or neighboring plants by change in spectral quality of light; and direct effect of light on plant development, known as photomorphogenesis.

Physiological and biochemical evidence gathered during the past several years has indicated that plants have a capacity to detect different spectral qualities of light, which are likely to be perceived by several distinct photoreceptors. It is now believed that plants use at least three groups of photoreceptors: UV-B photoreceptors (280-320 nm), Blue/UV-

A photoreceptors (320-500 nm), and phytochromes (600-750 nm). Current evidence favors the view that within a group there are likely to be multiple photoreceptor species, which may cooperatively or independently carry out the task of perception of light. Among these three groups the molecular identity of UV-B photoreceptor(s) is still unknown; for one of a blue/UV-A photoreceptors, a gene encoding a putative photoreceptor candidate has been cloned. At the biochemical level only the phytochrome has been purified; genes encoding for it were cloned from several plant species. Phytochrome is a chromoprotein with a single linear tetrapyrrole as a chromophore. It exists in two forms, Pr and Pfr, with absorption maxima in the red (660 nm) and far-red (730 nm) regions respectively. These forms are photoconvertible to each other.

It has been shown that several light-controlled metabolic and developmental processes in plants are initiated after perception of light by the phytochrome, and that many of these photoresponses ensue from alteration in the expression of specific genes. Since biochemical evidence indicates that phytochrome is likely to be a cytosolic protein, it is apparent that phytochrome regulation of nuclear gene expression involves intermediates which can probably move in and out of nuclei to carry the signal. In addition, several distinct intermediates may perhaps be involved since it is now known that there are many phytochrome species. Though available evidence indicates the existence of such intermediates in a phytochrome-triggered signal chain, the nature of the participating intermediate molecules and the mode of signal transmission has been only partly deciphered.

Recent molecular-genetic approaches using photomorphogenic mutants with a combination of physiological, biochemical, and molecular analyses have greatly added to our current understanding of phytochrome and its functions. These approaches have provided insight into photoperception and the possible identity of the intermediates of signal transmission. Broadly, two class of mutants have been obtained: mutants defective in the perception of the light signal, and mutants where a photomorphogenesis-like developmental program is triggered even in the absence of light signals. These mutants have now been reported in many species like *Arabidopsis*, tomato, sorghum, pea, tobacco, etc.

Among these mutants the tomato *aurea (au)* mutant was first to be analyzed at the biochemical and molecular-genetic levels. It was found that while the *au* mutant possesses no spectrally active phytochrome in etiolated seedlings, it is able to complete its life cycle normally, and retain several phytochrome responses. Among the several phytochromes likely to be, present, in the *au* mutant, the level of phytochrome A especially is severely depleted. This phytochrome deficiency is perhaps because of the *au* possessing a defective chromophore biosynthetic pathway. Though the level of other phytochrome species might also be low, the *au* possesses at least one spectrally active phytochrome species in the green tissues, which is probably phytochrome B. Deficiency of phytochrome, and the associated responses in young seedlings in the *au* have been exploited to develop single-cell assays for microinjection of phytochrome in epidermal cells to restore photomorphogenesis at the cellular level.

In the present study, we have used phytochrome-deficient etiolated *au* seedlings to decipher the operation of certain metabolic pathways such as anthocyanin induction, nitrogen metabolism, and Chloroplast development, by studying induction levels of certain key enzymes participating in these pathways. We compared the above processes with a wild type tomato, and a mutant with exaggerated photomorphogenesis such as a high pigment *{hp)* mutant. Our studies show that in spite of being deficient in phytochrome, *au* seedlings show photoinduction of cytosolic enzymes under continuous RL. However, the same seedlings are deficient in inducing plastidic enzyme and Chloroplast development.

2. REVIEW OF LITERATURE

2.1 Mutants as an aid to the study of plant growth and development

To offset the disadvantages of being sessile organisms, plants evolved, during the course of evolution, several sensitive mechanisms to perceive environmental changes, and to elicit adaptive responses for survival. These mechanisms enable plants to adapt physiologically and biochemically for short periods, while an altered growth and development program ensures long-term adaptation to ambient environment. In addition to its environmental regulation, plant development has unique features such as an extraordinary plasticity, an open-ended type of development, totipotency of cells, and production and termination of resting stages during the life cycle.

Plant development is governed by an interplay of several endogenous signals such as plant growth hormones and environmental stimuli such as light, temperature, etc. Among the environmental factors, light influences several facets of development throughout the life cycle of plants, right from the release of dormancy to plant senescence. Plants have evolved specialized mechanisms to elicit information about their light environment: detection of direction of light, onset of dusk and dawn, duration of photoperiod, shading of plants, reflection of light from neighboring plants, etc. To carry out the above tasks, plants have also evolved specialized photoreceptor(s) to sense light environment either cooperatively or independently. To date three photoreceptor groups have

been identified from studies involving action spectroscopy of photoresponses: phytochrome, blue/UV-A receptor, and UV-B receptor which cover different regions of the light spectrum.

The molecular nature of photoreceptors perceiving the light signal and the components participating in signal transmission remain largely unknown, except for phytochrome which is the only biochemically identified photoreceptor in plants. Though conventional biochemical, physiological, and molecular biological approaches have uncovered general routes of signal transmission and control of gene expression, these approaches suffer from a lack of specific identification of the participating molecules. The advent of recent technology to raise transgenic plants, and select and generate single mutations with specific phenotypes, has greatly amplified the knowledge about plant development and metabolic pathways. For a few mutants, the responsible genes have also been cloned; in many instances these genes appear to code for novel proteins as components of signal transmission or perception pathway. A combination of molecular biology and genetics with phenotype changes detectable by physiological behavior have indicated the putative role of these mutants in regulation of development.

The potential benefits derived by use of photomorphogenic mutants to probe the perception and transmission pathway is summarized below:

1) Information about the role of light in plant development can be obtained by phenotypic characterization of mutants.

2) These mutants also provide information about photoreceptor/ chromophore synthesis and action.

3) The mutants can be used to isolate and characterize genes encoding for photoreceptor and components of signal transmission chains.

4) Mutants can be used as test plants to make *in vivo* analysis of modified and chimeric phytochrome species and protein encoding components of signal chain.

During the past five years photomorphogenic mutants have revealed that components of the signal transduction pathway emanating from photoreceptors, consist of several novel proteins, and that some of these signal components may be similar across divergent groups of organisms.

2.2 Photomorphogenic mutants

Geneticists and breeders isolated several mutants, many of which turned out to be photomorphogenic mutants in eventual analysis. However an intensive and a specific search for photomorphogenic mutants was first initiated by Koornneef et al., (1980), who isolated six mutants from mutagenized seeds of *Arabidopsis,* using a phenotype based screening, i.e., retention of etiolated phenotype with elongated hypocotyl under continuous WL in seedlings. He named them *hy1 to 6. A* reverse of Koornneef's technique was used by Chory (1989a) to isolate mutants with a light-grown phenotype in plants grown in darkness. These strategies have led to the isolation of several mutants which are either deficient in photoreceptors or components of the signal transduction chain.

Most of the mutants isolated belong to phytochrome; a few to BL, and perhaps very few to UV-B light. Table 2.1 summarizes the present available mutants in various species. The present review, however, deals mainly with phytochrome-related photomorphogenic mutants.

2.2.1 Phytochrome mutants

Phytochrome is a chromoprotein with a linear tetrapyrrole as a chromophore; principally it detects the ratio of red and far-red light available to plants. The relative amounts of R/FR light are detected using a characteristic feature of the phytochrome molecule, namely, that it can exist in two forms: namely Pr (λ_{max} 660 nm) and Pfr (λ_{max} 730 nm), which are interconvertible by exposure to RL and FRL. However, it was believed till a decade ago that phytochrome exists as a single molecular species. The diverse influence of light signals and the spectral dependence of photoresponses indicated to photophysiologists the possible involvement of more than one phytochrome in mediating these responses (Hillman 1967, Smith and Whitelam 1990, Smith 1992). Parallel physiological, biochemical, and gene cloning studies revealed that phytochrome is encoded by a small multi-gene family (Furuya 1993), the members of which can be classified broadly into two classes on the basis of their respective susceptibility to *in vivo* exposure to light (Furuya 1989). Type-I, or light labile phytochrome, is characterized by strong accumulation in etiolated seedlings or dark-adapted tissues, but a severe down regulation of protein level in light-grown plants/tissues (Furuya 1991). Only one phytochrome species, namely phyA, belongs to this category. On the other hand, type-II, or light-stable species, are

Table 2.1: *List of available photomorphogenic mutants in higher plants*

Loci	Arabidopsis	Tomato	Tobacco	Pea	Cucumber	Sorghum	Brassica
PHYA	*fhy-2, hy8, fre-1*	*fri, au-?*	*pew*				
PHYB	*hy3 (Phyb-1, Phyb-2)*	*tri*			*lh*	ma_3^R	*ein*
Chromophore biosynthesis	*hy1, hy2, hy6*	*au-?*	*pew-1*				
Signal chain	*hy5, fhy1-1, fhy3-1, det1 to det3, cop1 to cop4, cop8 to cop11, doc1 to doc3, cue*	*hp*		*lip , lv*			
Blue/UV-A	*hy4, blu1 to blu4*						
Phototropism	*JK345, ZR8, ZR19, JK218, JK224, JK229, NPH1 to NPH3, rpt1, rpt2*						
UV-B responses	*uvr, uvh*						

relatively unaffected by the presence or absence of light *in vivo* and comprise several phytochrome species.

The first direct evidence for the presence of structurally distinct phytochrome molecules in same plant species came from immunochemical and spectroscopic studies in *Avena,* using polyclonal and monoclonal antibodies of different pools of phytochrome from etiolated and green *Avena* shoots (Shimazaki and Pratt 1985, 1986; Tokuhisa et al., 1985). A detailed molecular genetic analysis of phytochrome genes in *Arabidopsis* (Sharrock and Quail 1989) revealed at least five divergent gene sequences (Sharrock and Quail 1989, Clark et al., 1994): *PHYA, PHYB, PHYC, PHYD* and *PHYE.* All of these were isolated and sequenced. The amino acid sequence revealed that *PHYA* belongs to type-I category and is closely related to other forms isolated from oat, zucchini, pea, maize, and rice, which encode the phytochrome that accumulates to high levels in etiolated seedlings. In contrast, *PHYB-E* belong to type-II light-stable phytochromes encoding apoproteins PHYB through PHYE. Except for PHYB and PHYD which share 80% amino acid sequence identity, pairwise comparisons between any two *Arabidopsis* PHY reveal identities of from 46 to 55% (Pratt 1995).

Similarly, in tomato five *PHY have* been sequenced and compared to the five *Arabidopsis PHY* (Pratt 1995). These comparisons have identified a *PHYA,* two *PHYB (PHYB1* and *PHYB2),* a putative *PHYE* and a *PHYF* (No sequence similarity to any known *PHY,* hence named *PHYF).* The results obtained from low stringency genomic southern blots prepared with DNA digested with five endonucleases and probed with

fragments of tomato *PHYA*, *PHYB1*, *PHYB2*, *PHYE* and *PHYF* and of *Arabidopsis PHYC*, indicate that there are at least four and as many as eight additional *PHY* (Pratt 1995). In sorghum, three *PHY* (*PHYA*, *PHYS* and *PHYC)* have been identified by sequence comparison of PCR fragments to tomato *PHY* (Cordonnier-Pratt et al., 1994) However, low stringency genomic southern blots probed with tomato *PHYE* and *PHYF* indicated presence of additional *PHY* in sorghum (Pratt 1995). In rice also, both *PHYA* and *PHYB* have been sequenced (Kay et al., 1989: Dahesh et al., 1991) and the presence of a *PHYC* has also been reported (Quail et al., 1991).

The existence of multiple molecular species of phytochrome has triggered off a keen interest in learning the varied roles the different phytochrome forms might play in controlling photomorphogenesis. Are they functionally redundant or does each phytochrome perform a specific photobiological function, with a distinct molecular mechanism of action? Efforts are currently underway to answer these questions as several phytochrome mutants have been recently isolated which lack a given phytochrome species. Since phytochrome is a chromoprotein, phytochrome mutants exist in two classes, mutants with altered phytochrome gene or altered chromophore synthesis.

2.2.1. J Phytochrome gene mutants

Although multiple apophytochrome encoding genes have been identified in higher plants, only phytochrome A and phytochrome B deficient mutants have so far been isolated.

2.2.1.1.1 PhyA-deficient mutants

Among phytochrome mutants, phyA-deficient mutants have been isolated only recently from *Arabidopsis*, tomato, and tobacco. Three laboratories have simultaneously reported *Arabidopsis phyA* mutants. They are *fre1-1*,*fre1-2* (Nagatani et al., 1993), *fhy-2* (Whitelam et al., 1993), and *hy8-3* (Parks and Quail 1993). These mutants were isolated by adopting a screening strategy based on the assumption that responses of the etiolated seedlings to continuous far-red light are mediated by phyA (Smith and Whitelam 1990). Accordingly mutants were isolated which displayed elongated hypocotyl in continuous FR, but not in continuous R or WL. Molecular analysis of the above mutants revealed that these mutants lack spectrophotometrically and immunochemically detectable phytochrome A, possess reduced or undetectable levels of PHYA transcript, and have structural alterations of the PHYA gene (Whitelam et al., 1993). However, under white light, these mutants exhibit a morphology similar to WT, and complete their life cycle normally without showing adverse effects on the plant phenotype. (Nagatani et al., 1993; Parks and Quail 1993).

In contrast to above mutants, a new locus was identified in tobacco. Mutants at this locus exhibit long hypocotyls and chlorotic cotyledons under WL (Kraepiel et al., 1994); they were named *pew2* (partly etiolated in WL). Further analysis of mutants at *pew2* locus revealed that they contain no photoreversible phytochrome in etiolated seedlings, but possess normal levels of photoreversible phytochrome when grown in WL. Biliverdin has no effect on rescue of light-regulated responses in this mutant, except for *pew1* which is chromophore

deficient. Based on the above data it is proposed that *pew2* mutant could be affected in the *PHYA* gene itself.

Among these mutants, *hy8-3* mutant requires special mention, for it accumulates a mutant phyA molecule which has a normal spectral activity, but lacks biological activity, and fails to show inhibition of hypocotyl elongation in FR. This mutant is a potential candidate for the identification of regions on the phyA molecule responsible for transmitting the light signal to the next component of the signal transduction pathway (Parks and Quail 1993). Recently, tomato mutants specifically deficient in phyA have been isolated (Van Tuinen et al., 1995a, b). Like *Arabidopsis* mutants, tomato *PhyA* deficient mutants also have a phenotype similar to that of WT plants when grown in WL. (Tomato mutants will be discussed in detail in a separate section).

2.2.1.1.2 PhyB-deficient mutants

The availability of monoclonal antibodies against PHYB in 1991 made it possible to confirm that plants displaying elongated phenotype and an aberrant or missing EODFR response are in fact phyB-deficient mutants. In contrast to phyA, several phyB-deficient mutants have been isolated: *hy>3* mutants of *Arabidopsis* (Nagatani et al., 1991; Somers et al., 1991; Reed et al., 1993), the *lh* mutant of cucumber (López-Juez et al., 1992), the *ein* mutant of *Brassica rapa* (Devlin et al., 1992), the ma_3^R mutant of sorghum (Childs et al., 1991, 1992), and the *tri* mutant of tomato (Van Tuenin et al., 1995b).

Unlike phyA-deficient mutants, phyB-deficient mutants display an elongated hypocotyl in R and WL but not in FR (Adamse et al., 1987;

Koornneef et al., 1980; Devlin et al., 1992). Among these, *lh*, *hy3* and *ein* mutants display a light-dependent pleiotropic phenotype that is similar to the low R/FR ratio induced shade avoidance syndrome. Further these mutants also show early flowering compared to their isogenic WT (Kendrick and Nagatani 1991; Nagatani et al., 1991; Whitelam and Smith 1991). In contrast, ma_3^R does not show greater elongation growth of the stem but shows photoperiod insensitivity in sorghum which is a short day plant (Childs et al., 1991). Immunochemical analysis has shown that these mutants are deficient in phytochrome B (Devlin et al., 1992; 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).

A number of *hy3* alleles of *Arabidopsis* are null mutants of phyB (Reed et al., 1993). Three of these alleles have stop codons in the *PHYB* coding sequence, one has a missense mutation at amino acid residue 283 that causes a histidine to tyrosine change, suggesting that the *hy3* phenotype is due to a deficiency of functional phyB. Further studies have shown that elongation of many tissue types is effected throughout the development of this mutant (Reed et al., 1993). Observations of cotyledons of intact *hy3* seedlings suggest that phyB is involved in light-driven cotyledon expansion (Neff and Von Volkenburgh 1994); results from more recent experiments demonstrate that phyB is necessary for light stimulated cell expansion in cotyledons of *Arabidopsis* (Neff and Volkenburgh 1994).

Brassica ein and sorghum ma_3^R mutants were originally identified to be gibberellin over-producing than corresponding WT plants. Furthermore, GA metabolism was shown to be influenced by light in dark-grown seedlings (Sponsel 1986), and GA biosynthesis was suggested to be under phytochrome control during de-etiolation in pea (Campell and Bonner 1986), after R or FR pulse in light-grown cowpea (Garcia-Martinez et al., 1987), suggesting the role of GA in phytochrome action and vice versa. Experiments with the *phyB*-deficient *lh* mutant of cucumber which show an increased responsiveness to applied GA compared to its WT (López-Juez et al., 1995), suggest that GA4 and phytochrome control cell elongation through separate mechanisms that interact a step closer to the terminal response (López-Juez et al., 1995).

2.2.1.1.3 Chromophore-deficient mutants

Arabidopsis hyl and *hy2*, and tobacco *pew 1* (Kraepiel et al., 1994) are the only true chromophore mutants identified so far in higher plants. Recent data suggest that the *Arabidopsis hy6* and tomato *aurea* mutants are defective in chromophore biosynthesis (Reed et al., 1992; Whitelam and Harberd 1994).

hyl, hy2 and *hy6* mutants deficient in phytochrome mediated de-etiolation, display a dramatically elongated hypocotyl in WL, RL, and FR, and a slightly elongated hypocotyl in BL. The adult mutant plants are paler than the WT (Koornneef et al., 1980; Chory et al., 1989a). It has been reported that *hyl, hy2*, and *hy6* mutants have greatly reduced levels of spectrophotometrically detectable phytochrome, but have WT levels of immunochemically detectable PHYA (Chory et al., 1989a; Parks et al.,

1989). These observations suggest that the *hyl, hy2,* and *hy6* mutants have normal levels of phytochrome apoprotein in the dark, but, it is photochemically nonfunctional. In subsequent experiments *hyl* and *hy2* mutants were grown in light on a medium containing biliverdin IXα (a precursor of phytochrome chromophore phytochromobilin) in which they displayed a phenotype indistinguishable from that of light-grown WT plants and had WT levels of photochemically active phytochrome. This indicates that these mutants are blocked at a stage of phytochromobilin biosynthesis prior to biliverdin IXa (Parks and Quail 1991).

Since all phytochromes in a given plant probably use the same chromophore, these mutants may be deficient in more than one form of phytochrome. However, the mutants do not absolutely lack phytochrome activity. A low level of spectrally active phytochrome is detectable (Chory et al., 1989a; Whitelam and Smith 1991) along with a reduced level of R/FR reversible *CAB* gene expression (Chory et al., 1989a) which is phytochrome mediated (Karlin-Neumann et al., 1988). This observation suggests that the above mutants are partially rather than completely phytochrome deficient, indicating that they may be leaky mutants or there might be more than one pathway for pyrrole biosynthesis, or that they might be producing partly spectrally active phytochrome species in different amounts.

Similarly, *pew 1* mutants of tobacco exhibiting long hypocotyls and chlorotic cotyledons under WL contain normal levels of immunochemically detectable phytochrome in etiolated seedlings, and deficient in spectrophotometrically detectable phytochrome whether the

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Similarly, *pew 1* mutants of tobacco exhibiting long hypocotyls and chlorotic cotyledons under WL contain normal levels of immunochemically detectable phytochrome in etiolated seedlings, and deficient in spectrophotometrically detectable phytochrome whether the

seedlings are grown in dark or light. Moreover, as in *hyl* and *hy2* mutants, biliverdin restores light-regulated responses in *pew I* mutants and increases photoreversible phytochrome when grown in the dark indicating that the *pew J* locus may be involved in chromophore biosynthesis (Kraepiel et al., 1994).

2.2.1.2 Pr form of phyB is also physiologically active

Recent data on shoot gravitropism (Liscum and Hangarter 1993), and germination responses (Reed et al., 1994, Shinomura et al., 1994) obtained by using *Arabidopsis* mutants *hyl* (chromophore deficient), *hy3* (phyB-deficient), and *hy8-2* (phyA-deficient) indicates that in addition to the Pfr form, the Pr form of phyB is also active. It was found that WT seedlings grew upright in the dark, and randomized in RL, and phyB mutants grew randomly in both dark and RL, indicating that Pr form of phyB promotes correct gravitropism in the dark. In contrast, chromophore-deficient *hy2* mutant, in which phyB is locked in the Pr form grew upright in both RL and dark, suggesting that the RL-absorbing Pr form of phyB promotes negative gravitropism. Studies on germination response of *phyB*-deficient seeds have led to a similar conclusion i.e., *phyB* mutant seeds germinate better in FRL than WT seeds, indicating that the Pr form of phyB must be inhibiting germination; experiments with RL suggested that Pfr form of phyB promote germination. In contrast, there is no evidence to this date of the activity of the Pr form of phyA.

2.2.1.3 Signal-transduction mutants

Mutants that contain a particular photoreceptor, but lack all photomorphogenic responses associated with it in light (Chory 1992; Whitelam et al., 1993), and the mutants that display the phenotype of light-grown plants even in the dark (Chory et al., 1989b; Deng et al., 1991) are the most probable candidates for signal transduction chain mutants. Mutants which fit into the first group are the three elongated hypocotyl conferring loci of *Arabidopsis, hyS, fliyl,* and *fliy3* (Chory 1992; Whitelam et al., 1993), and the *lv* mutant of pea (Nagatani et al., 1990). Mutants which fit into the second group are three *det* (de-etiolated) (Chory et al., 1989b), and eight *cop* (constitutive photomorphogenesis) (Deng et al., 1991) loci *of Arabidopsis*, and a *lip* mutant of pea (Frances et al., 1992).

2.2.1.3.1 Mutants with strong dark morphology in light

The *hy5* mutant *of Arabidopsis* displays an elongated hypocotyl in R/FR, and has reduced sensitivity to BL, but has normal levels of phytochrome (Koornneef et al., 1980). Though the molecular lesion in *hyS* mutants is not known, a *hyS* mutation in combination with either *hyl, hy2* (chromophore mutants) or *hy3* (PhyB mutant) has an additive effect on inhibition of hypocotyl elongation (Koomneef et al., 1980), indicating that the *HYS* locus may encode a signal transduction component downstream of multiple phytochromes, and of the blue light receptor.

The phenotype of *fhy1* and *fhy3* mutants resembles that of phyA-deficient mutants, by displaying an elongated hypocotyl in FR but not in

R or WL, but it has normal levels of spectrophotometrically and immunochemically detectable phytochrome A (Whitelam et al., 1993). However, the *fhy1-1* and *fhy3-1* mutations complement the phyA mutations and the *FHYl* locus has been shown to be genetically unlinked to *PHYA.* Therefore it is likely that *fliyl-1* and *fltyS-J* mutants identify genes whose products are involved in a phyA signal-transduction pathway.

The *lv* mutant of pea exhibits a phenotype similar to PhyB-deficient mutants; with an increased internode length in the light, it lacks EODFR response on stem elongation, and has a retarded de-etiolation in light. Spectrophotometrical and immunochemical analysis revealed, that both etiolated and light-grown tissues of the mutant contain normal levels of PhyA and PhyB. Hence it was proposed that *lv* may be a signal-transduction mutant (Nagatani et al., 1990). Investigation into the mechanism of internode elongation in the mutant has revealed that *Iv* mutation does not decrease the levels of gibberellin like substances or increase the metabolism of applied gibberellin A_{20} (Reid and Ross 1988). Furthermore, there is no evidence that the rate of GA_1 production or GA_1 metabolism are important determinants of stem elongation in pea. However, it was shown that etiolation and the *lv* mutation both increase the responsiveness of the WT plant to GA] (Weller et al., 1994). Interaction between light treatment and the *lv* mutant is consistent with the hypothesis that light acts in part by constraining GA-signal transduction at a relatively late stage (Nick and Furuya 1993). It was also observed that the constitutive high growth rate in *lv* plants was accompanied by a 25% or more increase in the extractable amounts of IAA from

epidermal peels in comparison to isogenic WT (Behringer et al., 1992). It is therefore possible that the /v mutation causes increased internode growth in part by blocking the ability of phytochrome to decrease epidermal IAA levels.

2.2.1.3.2 Mutants with partial/complete light morphology in dark

The second group of signal transduction chain mutants includes eleven loci in *Arabidopsis* and one in pea; these show a complete or partial light-grown phenotype even when the seedlings are grow in dark. Among these the dark-grown *det J, cop J, cop8, cop9, cop 10, cop J 1*, and *lip* mutants display the most pleiotropic phenotype including cell differentiation patterns, plastid differentiation, and gene expression (Chory, 1989a and 1989b; Deng et al., 1991; Deng, 1994; Frances et al., 1992). on the other hand, *det2, det3, cop2, cop3,* and *cop4* mutants exhibit only subsets of light responses when grown in darkness. Therefore, it is likely that the most pleiotropic loci define signaling steps in the same pathway after the convergence of the signals from different photoreceptors, before the branching of the pathway into individual responses. Further the less pleiotropic loci may define parallel or branched downstream pathways controlling individual responses of seedling development like the *COP4* locus which may be involved in both the light signaling and gravity sensing processes. In other words *DET2, COP2, COP3, COP4* and *DET3* loci are involved in a subset of functions, whereas *DET1, COP 1, COP8, COP9, COP 10* and *COP 11* may be involved in controlling the primary switch between the two basic

developmental programs, i.e, photomorphogenesis and skotomorphogenesis.

2.2.1.3.3 Hierarchial positions of different loci

Epistatic analysis of double mutants between *cop, def* or *hy5* mutations and the mutations affecting photoreceptors suggested that DET1, COP1, COP8, COP10, COP11 and HY5 act downstream of both phytochromes and a blue light receptor (Chory 1992; Ang and Deng 1994; Misera et al., 1994; Wei et al., 1994) by repressing photomorphogenesis, i.e., photoreceptor activation reduces the activity of these negative regulators allowing photomorphogenesis to ensue; the exception is *hy5*, which has been identified to be an activator rather than a repressor of photomorphogenesis. Similar analysis also suggested the hierarchial positions of these loci, which are in the order of *COPl, COP8, COP9, COP10* and *COP 11* downstream of *HY5* and the *DET1* act either in an independent pathway or upstream of *HY5* (Fig 2.1).

However, it is difficult to say specifically that *del* and *cop* mutants are photomorphogenic signaling mutants, due to the complexity of the photomorphogenic developmental program, where photomorphogenic traits can be induced by multiple stimuli. For example the *det1* phenotype can be induced in WT seedlings by growing them on cytokinin (Chory et al., 1991, 1994). Light-induced genes like Lhcb can be induced even in the dark, in response to factors such as cyclic temperature variation (Brusslan and Tobin 1992, Kloppstech et al., 1991). Also, the six of the eleven constitutive mutants characterized to date *(detl, cop1, cop8, cop9, cop10, and cop11)* were shown to be allelic

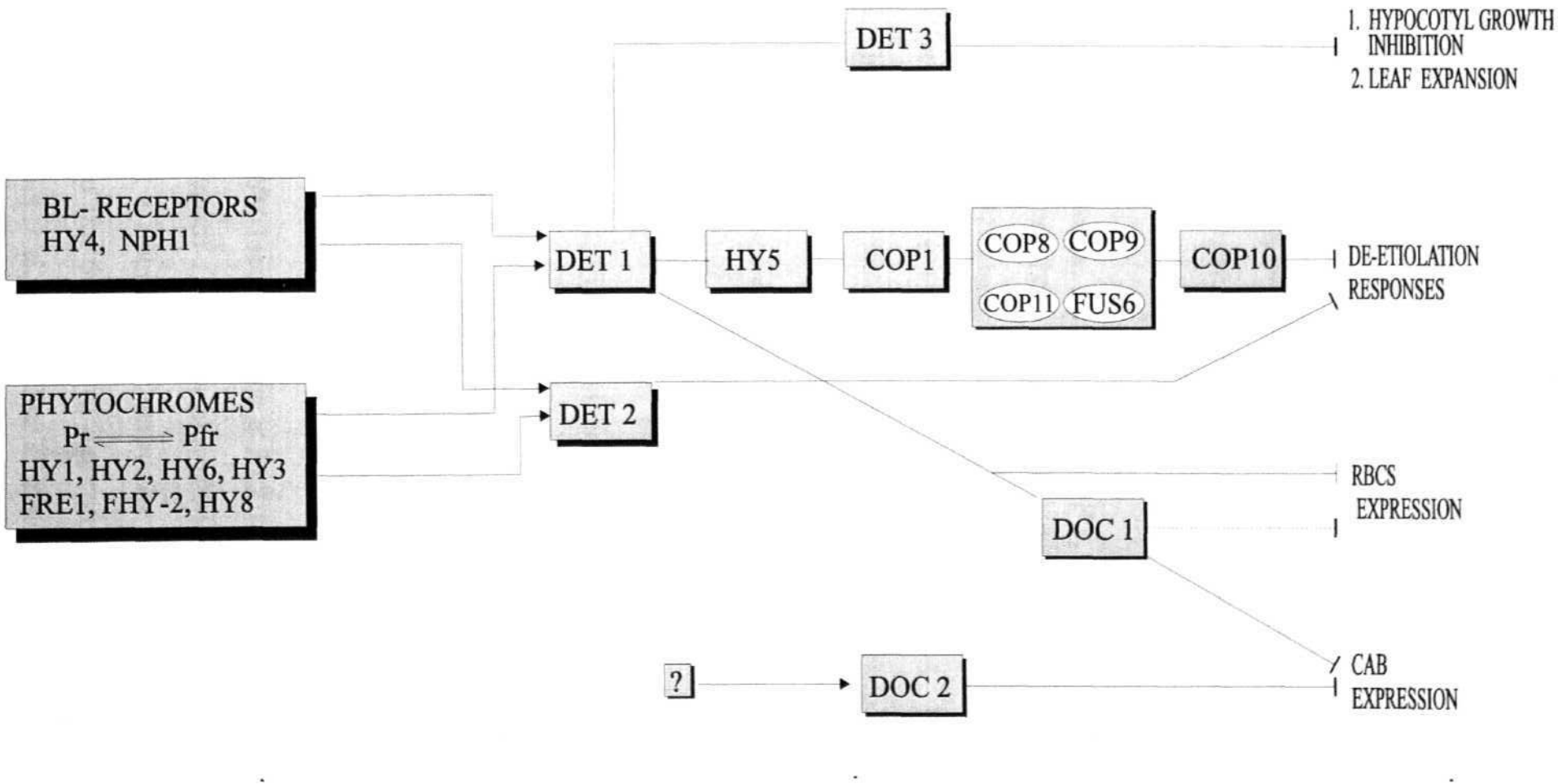

Figure 2.1 Genetic model of signal transduction chain involved in *Arabidopsis* seedling development. (Adapted from Chory, 1992, Li et al., 1994)

to previously described seedling lethal mutants (*fus2, fus1, fus8, fus7, fits9, and fuss)* known as *fusca* mutants (Castle and Meinke 1994, Misera et al., 1994). The above observations imply that *COP* and *DET* products are not just specific to phytochrome signaling, but act as negative global regulators for plant development, which are able to receive signals from multiple stimuli and integrate them into a developmental response.

2.2.1.3.4 Structural analysis of COP1, COP9, COP11 and DET1

Currently we have information regarding four of these negative regulators: COP1 (Deng et al., 1992), COP9, (Wei et al., 1994), COP11 (Castle and Meinke 1994), and DET1 (Pepper et al., 1994). Molecular cloning and sequence analysis of these loci have revealed the structural features which are very much inconsistent with their proposed functional roles. However, the nature of their involvement in signal transduction is yet to be deciphered. The *DET1* locus encodes a 62.2 kD protein containing two regions (Pepper et al., 1994) that are similar to known bipartite nuclear localization signals (Robbins et al., 1991). The DET1 protein has been physically shown to be nuclear localized, suggesting that it acts in the nucleus to control the cell type specific expression of light regulated promoters. *det1* null mutants display severe defects in temporal and spatial regulation of gene expression.

The *COPl* locus encodes a 76.2 kD protein with a ringer finger Zinc binding motif (Boddy et al., 1994), and coiled coil motif at N-terminus, and a domain at C-terminus with multiple WD-40 repeats homologous to the ß-subunit of heterotrimeric G-protein (Deng et al., 1992, McNeillis et al., 1994). The above structural feature indicates that

COP1 has the ability to bind DNA directly through it's Zn binding domain, and that it is capable of interacting with other protein components of the light signaling network through its G-protein homologous domain. It was shown that the C-terminus portion of COP1 has homology to Drosophila $TAF_{II}80$ protein, a subunit of the TFIID complex that is required for RNA polymerase II transcription initiation, suggesting a role for COP1 in repressing general transcription. This is in agreement with it being proposed as a negative global regulator for plant development. The recent results of Von Arnim and Deng (1994) suggest that COP1 is localized to the nucleus in darkness and its nucleocytoplasmic partitioning is regulated by light in a cell type specific manner through light-triggered nuclear depletion of the protein.

COP9 encodes protein of predicted molecular mass of 22.5 kD whose amino acid sequence does not bear significant homology with other protein sequences in the database. It is proposed that COP9 functions as part of large >560 kD complex and require COPS and COP11 products for either formation or stability of this complex (Wei et al., 1994). COP 11 possesses a protein kinase C phosphorylation site suggesting that at least these three gene products act together in a network of signal-transduction pathways critical for plant development.

In addition to the above two groups of mutants which were isolated using morphological screens, a new class of mutants were isolated using a newly designed screen based on the aberrant expression of a light-regulated hygromycin resistance gene to improve specificity. They are the *doc* mutants (Li et al., 1994), which overexpress the *CAB* genes in the dark, and *cue* mutants, in which the *CAB* promoter is

underexpressed in the light (Chrisspeels et al., 1995), and *GUN* mutants, which exhibit nuclear gene expression of plastidic proteins in the absence of Chloroplast development, and may interfere with the switch from dark-grown to light-grown development (Susek et al., 1993).

2.2.1.4 Transgenic mutants

Development of transgenic mutants is another important strategy employed in the current investigations of photomorphogenesis, e.g., for assigning roles to multiple phytochrome species, and for their structure/function analysis. Basically two simple experimental approaches have been adopted. The first is the development of transgenic mutants that are deficient in the presence or activity of particular phytochrome species by creating dominant negative mutations (Herskowitz 1987) using either antisense RNA or antisense protein techniques. The use of antisense RNA techniques to create phytochrome mutations have not been reported. The antisense protein approach was employed through intracellular expression of a recombinant antibody derivative specific to phytochrome A in transgenic plants. Though the phenotype of transgenic plants was not different from that of WT, the transgenic seeds displayed retarded germination in response to an inductive light pulse (Owen et al., 1992).

The second approach, involving overexpression of individual phytochrome species, has been employed in most studies. Here it is expected that the photoresponses initiated by the overexpressed phytochrome species may be exaggerated, thus allowing the responses that are regulated by that phytochrome species to be identified. Such

overproducing mutants can be classified into three groups: phytochrome overexpression in WT, phytochrome overexpression in photomorphogenic mutants and mutated phytochrome overexpression in WT.

2.2.1.4.1 Phytochrome overexpression in wild type

Only phyA and phyB overexpressing mutants have so far been reported in the literature. The overexpression of cloned *PHYA* cDNA in heterologous transgenic plants has been reported from several laboratories. Transgenic plants expressing introduced cereal *PHYA* cDNA under the control of cauliflower mosaic virus 35S promoter have been generated in tomato, tobacco, and *Arabidopsis.* In all these cases, transgenic plants display a light-conditional dwarf phenotype indicating biological activity of the transgene product. They also exhibited one or more of the following phenotypic characteristics: increased pigmentation, increased branching, or reduced leaf senescence (Boylan and Quail 1989; Keller et al., 1989). Similarly, *Arabidopsis* or rice *PHYB* cDNA is expressed in *Arabidopsis* under the control of cauliflower mosaic virus 35S promoter. These seedlings also display a light-conditional dwarf phenotype (Wagner et al., 1991; McCormac et al., 1993a) indicating that both phytochrome A and phytochrome B action impinges upon the same cellular process such as stem elongation.

Physiological analysis of etiolated transgenic seedlings of tobacco and *Arabidopsis* transformed with oat *PHYA* cDNA, and *Arabidopsis* seedlings transformed with *Arabidopsis* or rice *PHYB* cDNA, revealed that phytochrome A is the photoreceptor mediating FR high irradiance

response; that this is the innate property of phytochrome A, and is not mediated by phytochrome B. Additionally, photoresponses that are absent in mutant seedlings that are null for a particular phytochrome are reinstated in the transgene seedlings overexpressing the same phytochrome species. For example, phyB-deficient *hy3* seeds poorly germinate in the dark, but *phyB* transgenic *Arabidopsis* seeds show very high dark germination levels (McCormac et al., 1993b). Similarly, phyA-deficient *Arabidopsis* seedlings are late flowering under extended short day conditions, but *phyA* transgenic are early flowering under these conditions (Whitelam and Harberd 1994).

2.2.1.4.2 Overexpression of mutated phytochrome in wild type

Overexpression of mutated phytochrome gene sequences in transgenic plants has proved to be very helpful in structure/ function analysis of phytochromes (Fig 2.2). Here, the expectation is that mutations in the transgenic phytochrome, which eliminate the overexpression phenotype, will define functionally important regions of the molecule. The results obtained reveal that amino acids within 6-10 kD of the N-terminus are important to the physicochemical properties of phytochrome A. Deletion of this region results in alterations in spectral properties of phytochrome A, decreases the stability of Pfr, and increases the accessibility of the chromophore to chemical modifications (Vierstra and Quail 1986; Lagarias and Mercurio 1985; Cordonnier 1989). Site-directed substitution of Serine for Cysteine at the chromophore attachment site eliminates ligation and photosensory activity of both phyA and phyB (Quail et al., 1995). Domain swapping experiments in transgenic *Arabidopsis* have established that the N-terminal domain of

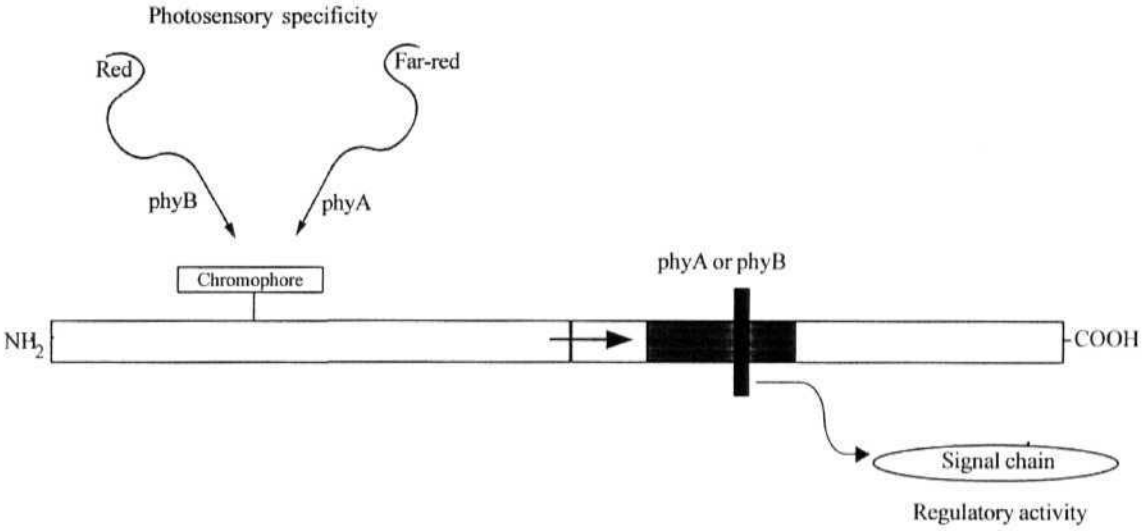

Figure 2.2: Different domains of phytochrome and their biological action. Arrow represents the intramolecular information transfer, shaded region is a 160 residues C-terminal segment required for successful transmission of the perceived signal to the downstream signal chain with indications that a subregion of 18 residues (solid) is particularly critical to this process (Adopted from Quail et al., 1995).

phyA is sufficient when fused to the C-terminal domain of phyB, to confer continuous FR photosensory activity on the chimeric molecule, but the C-terminal domain of phyA does not confer continuous FR photosensory activity on the N-terminal domain of phyB suggesting that, photosensory specificities of phyA and phyB towards continuous R and FR reside in the N-terminal domains (Quail et al., 1995). Amino acid residues between 7-69 of N-terminus of phyA are necessary for biological activity but not for dimerization, photochemical activity and *in vivo* down regulation of Pfr levels (Cherry et al., 1992). In contrast, the serine to alanine substitution of the first ten serine residues at the amino-terminal region of rice phyA was shown to increase the biological activity in the transgenic tobacco (Stockhaus et al., 1992). On the other hand, C-terminal deletion mutants of oat phyA revealed that, the amino acid residues between 399 and 652 are necessary for spectral integrity but not for chromophore attachment. Residues between 919 and 1093 are necessary for dimerization, and 35 residues from C-terminal end appear to be necessary for biological activity (Cherry et al., 1993).

2.2.1.4.3 Overexpression of phytochrome in phytochrome deficient mutants

This approach was adopted in the case of *aurea* mutant to resolve whether it is a chromophore-deficient or phyA-deficient mutant. Oat *PHYA3* gene was transformed into *au* line (McCormac 1993) resulting in the accumulation of *PHYA3* mRNA but not PHY A3 apoprotein, suggesting that the *au* mutation influences some process affecting mRNA translation or phytochrome protein stability. Later the oat *PHYA3* overexpressing line (Boylan and Quail 1989) was crossed with *au* and

phenotypically similar *yg2* mutant and *au,PHYA3* and *yg-2,PHYA3* double mutants were selected. Both transgenics accumulate PHYA3 apoprotein without any rescue of their mutant phenotype suggesting the deficiency in availability of the phytochrome chromophore.

Even though the transgenic mutants are often used in investigating and assigning physiological roles to a particular phytochrome species, there are three major drawbacks to be remembered while using this approach: first, it is difficult to say that the responses mediated by overexpressed phytochrome species are a true reflection of the same phytochrome species in WT; second, overexpressed phytochrome species may arrogate the normal function of the same or other phytochrome family members in the transgenic mutant; and finally, in the overexpression of mutated phytochrome, it is impractical to generate functionally important residues compared to conventional mutagenesis, and screening, which are capable of defining individual residues that are necessary for phytochrome function.

2.2.2 Blue/UV-A response mutants

Blue light and UV-A are known to stimulate several morphological and developmental changes in plants, such as inhibition of hypocotyl elongation, phototropism, apical hook opening, and opening of stomatal guard cells. Classical physiological experiments have indicated that these responses are mediated through the action of multiple photosensory systems. For example, many BL/UV-A responses, such as control of hypocotyl elongation, are also under the control of phytochrome. However isolation of *Arabidopsis* mutants which are

defective in BL-inhibited hypocotyl elongation (Koornneef et al., 1980; Liscum and Hangarter 1991; Nagatani et al., 1993), and phototropism (Khurana and Poff 1989; Khurana et al., 1989; Konjevic et al., 1992; Liscum et al., 1992; Okada and Shimura 1992; Liscum and Briggs 1995) have clearly shown the operation of a BL photoreceptor independent of phytochrome.

2.2.2.1 BL-mediated hypocotyl elongation mutants

So far, BL-mediated response mutants have been isolated only from *Arabidopsis*; they are the long hypocotyl *hy* and *blu* (blue light uninhibited) mutants. Among the different *hy* loci, *hy4* represents the first BL response mutant isolated from higher plants. The *hy4* mutant exhibits reduced growth inhibition compared to WT in high fluence rate BL, and a partial loss of growth inhibition in green and RL, where phytochrome is active (Koornneef et al., 1980; Ahmad and Cashmore 1993; Jenkins et al., 1993; Liscum and Hangarter 1994). However, *hy4* seedlings contain WT levels of phytochrome A, B, and C apoproteins (Somers et al., 1991), and normal levels of photoreversible phytochrome (Koornneef et al., 1980; Chory et al., 1989a) indicating that deficiencies observed in *hy4* stemmed from a lesion in a gene independent of those involved in biosynthesis of functional phytochrome. Recently, a t-DNA insertional mutant of *hy4* was isolated from which the *HY4* gene was cloned; its product is proposed as the putative BL photoreceptor (Ahmed and Cashmore 1993). The deduced amino acid sequence shows homology to microbial DNA photolyases with a putative flavine binding sites.

blu mutants (Liscum and Hangarter 1991) exhibit uninhibited hypocotyl growth in high fluence rate BL, and are genetically distinct from the *hy* mutants. They represent three additional loci, namely *blu J, blu2*, and *blu3.* Detailed analysis of the spectral and fluence rate dependencies of hypocotyl growth inhibition in the *blu* mutants showed that these mutants are defective specifically in their response to high fluence rate BL, while they retain WT response to UV-A, green, RL, FRL, and low-fluence rate BL. These results indicate that these loci affect a BL photosensory system independent of phytochrome. The independent action of the BL and phytochrome systems on hypocotyl growth inhibition can be well illustrated with the double mutants between BL response mutants and phytochrome-deficient mutants (Liscum and Hangarter 1991; Young et al., 1992; Liscum and Hangarter 1994). For example, *blu1,hy6* double mutants exhibit an etiolated phenotype in WL, BL, GL, and R/FRL. In contrast *blu1,hy8* double mutants have long hypocotyls only in BL and FRL. In addition to *blu* and *hy4* mutants thirteen mutant strains with BL-dependent hypocotyl phenotype, which is intermediate between WT and the *blu* or *hy4* mutants have been described (Liscum and Hangarter 1991; Jenkins et al., 1993). However, it remains to be determined whether these strains represent new loci.

Responses to UV-A light have generally been thought to involve the same photoreceptor that controls responses to BL, because many BL responses show maximal activities in both BL and UV-A light. The response in UV-A light has also been considered to be a high irradiance phytochrome response (Hartmann 1967). The spectral reactivities of

blu1, h\>6, and *blu1,hy6* double mutants clearly show that they retain a WT response to UV-A light. Hence, the response to UV-A light is not mediated exclusively by phytochrome, or a single B/UV-A light absorbing photosensory system, but involves the function of a third photosensory system with an absorption maximum in the UV-A region of the spectrum (Young et al., 1992).

2.2.2.2 Phototropic mutants

Phototropism is another important developmental response by which plants maximize their light capture for photosynthesis. This response shows spectral dependence on BL and UV-A light (Dennison 1979; Firn 1986). In order to understand the photoperception and signal-transduction mechanism mediating this response, 35 mutant strains that exhibit reduced phototropic curvature, relative to WT, in response to multiple flashes of unilateral BL have been isolated in *Arabidopsis* (Khurana and Poff 1989; Khurana et al., 1989); out of these, six strains were examined in detail. Three of these phototropism-mutant lines (JK345, ZR8 and ZR19) are altered in their gravitropic responses as well as their phototropic response (Khurana and Poff 1989; Khurana et al., 1989) indicating that these mutations might affect components involved in later steps of these signal response pathways, which is consistent with the fact that both responses require changes in cell elongation for phenotypic expression. The other three mutant strains (JK218, JK224, and JK229) are altered specifically in their phototropic response, but retain wild type gravitropic response (Khurana and Poff 1989). Therefore, JK218, JK224, and JK229 represent unique components of the phototropic response pathway.

In *Arabidopsis*, ased on the shape of fluence response curve for first positive phototropism which depends on fluence rate and wavelength of light used, Konjevic et al.,(1989) proposed that there are two photoreceptors PI and PII with absorption maxima at 450 nm and 510 nm respectively. This hypothesis was supported by the studies on JK224 mutant (Konjevic et al., 1992), which retained the fluence response curve similar to the WT at 510 nm, but not at 450 nm and led to the proposal that JK224 represent a PI photoreceptor mutant (Konjevic et al., 1992). It was also shown that JK224 is defective in a BL-dependent phosphorylation of a 124 KD plasma membrane protein (Reymond et al., 1992b), which was proposed to be an early step in a signal transduction pathway for phototropism (Short and Briggs 1990). But JK228, though it exhibits a null phototropic phenotype (Khurana and Poff 1989) it also shows a WT phosphorylation response, suggesting that mutation in JK218 represents a lesion in a signal transduction pathway.

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pathway for phototropism (Short and Briggs 1990). But JK228, though exhibiting a null phototropic phenotype (Khurana and Poff 1989), also shows a WT phosphorylation response, suggesting that mutation in JK218 represents a lesion in a signal transduction pathway.

Liscum and Briggs (1995) recently reported the isolation of eight mutants of *Arabidopsis* that lack or have a severely impaired phototropic response. These *nph* (non-phototropic hypocotyl) mutants comprise of four loci: *nph1, nph2, nph3*, and *nph4.* Physiological and biochemical characterization of the *nphl* allele indicates that the NPHl locus may encode the apoprotein for a dual-chromophore or multi-chromophore holoprotein photoreceptor capable of absorbing UV-A, UV-B, and GL, and regulates all the phototropic responses of *Arabidopsis.* It appears that NPHl protein is probably a 120 kD plasma membrane associated phosphoprotein. Furthermore, it was also shown that NPHl is genetically and biochemically distinct from the *hy4,* and that neither is functionally redundant.

Arabidopsis roots also show a negative phototropic response (Okada and Shimura 1992). To date, 97 putative mutant strains have been isolated with altered root phototropism, however, only two confirmed mutant lines designated *rptl* and *rpt2,* have been studied in detail. Although the *rptl* and *rpt2* mutants are defective in root phototropism, hypocotyl phototropism was found to be normal, as were root and hypocotyl gravitropisms, indicating that divergent pathways are operating for a variety of tropic responses.

2.2.2.3 BL-mediated signal transduction

A recent developn ent is the discovery of a rapid BL-induced phosphorylation of a plasma membrane protein (Gallagher et al., 1988). Biochemical and photobiological characterization (Short and Briggs 1990), and information from BL-response mutants (Reymon et al., 1992b) have implicated the phosphorylation of an approximately 120 kD protein as an early step in the control of phototropism. This reaction proceeds both *in vivo* and *in vitro* (Short and Briggs 1990; Short et al., 1992), and is functionally homologous among a broad range of species (Short 1991; Reymond et al., 1992a). *In vitro* phosphorylation of a protein in unirradiated membranes from one species with irradiated membranes to a different species suggested that light activates a specific kinase (Reymond et al., 1992a), although the identity of the kinase, and its relationship to the photoreceptor, is unkown. Similarly, the established signal-transduction components in other systems such as G-proteins, calmodulin, calmodulin-activated myosin light chain kinase like kinases, diacylglycerol, protein kinase C etc., have been implicated in BL-mediated responses like stomatal aperture, gene expression and stem elongation (for a review see Kaufmann 1993). However, all these components were not directly demonstrated together in any one response or system.

In summary, physiological analysis of the various BL mutants indicates that at least four distinct loci are involved in BL-dependent hypocotyl growth inhibition *{BLU1, BLU2, BLU3* and *HY4)* and at least three in phototropism (*JK218, JK224* and *NPHl).* If JK224, *NPH1* and *hy4* represent true photoreceptor mutants controlling phototropism and

hypocotyl elongation, then as in the phytochrome family, BL responses may also involve a family of photoreceptors with different functions.

2.2.3. Ultraviolet-B mutants

While studies with mutants in relationship to Blue/UV-A receptor are limited, still less is known about photoreceptor absorbing in the UV-B region of the spectrum. UV-B light is known to mediate photomorphogenic responses like phototropism (Baskin and lino 1987), hypocotyl growth (Ballare et al., 1991) etc., and flavonoid induction (Tevini et al., 1981, 1991; Beggs and Wellman 1985), along with DNA damage (Mclennan 1987; Pang and Hays 1991; Quaite et al., 1992), hormone inactivation (Tevini et al., 1991), Plasma membrane ATPase inactivation (Imbrie and Murphy 1984) and damage to photosystem II (Greenberg et al., 1989). Many physiological studies have clearly implicated the presence of a separate UV-B photoreceptor in a number of species (Wellmann 1971; Drum-herrel and Mohr 1981).

Although many plant responses to UV radiation have been reported, complete mechanistic details of most of the above responses have not been elucidated. One of the problems is that UV-B absorbed by the phytochrome also produces a significant amount of Pfr under continuous irradiation, making the interpretation of results from long-term experiments more difficult. Even the genetic studies aimed at understanding UV-B responses are limited. Mutants that are defective in one or more responses to UV radiation, and second site suppressors of these mutants will be very useful in defining the nature and signal-transduction pathway of the UV-B receptor. Two *Arabidopsis* mutants,

uvrl and *uvh1*, which are hypersensitive to UV-B radiation were recently reported (Britt et al., 1993; Harlow et al., 1994).

The *uvrl* mutant was isolated using a root bending assay (Britt et al., 1993) from EMS mutagenized *tt5* (Koornneef 1990) homozygous *Arabidopsis* plants. This mutant was six times more sensitive than its progenitor *tt5* to UV-B in elongation of immature root. Even the aerial tissues displayed a UV-sensitive phenotype along with the growth of the root tip. *uvrl* was shown to have a defect in the repair of common UV-B induced DNA lesion, i.e., formation of DNA photoproducts Pyramidine(6,4)Pyramidone dimers.

uvh1 was isolated using a newly developed screen in which test areas of leaf tissues were exposed to a low fluence UV while shielding the meristem which produces inflorescence then, the minimum UV fluence required to cause detectable wilting and chlorosis in leaves of WT plants was determined. The mutagenized seed stocks were screened for mutants which showed similar type of damage after exposure to several folds smaller fluence, which did not effect WT plants. UV-B damage to *uvhl* plants could be significantly reduced along with WT plants by subsequent exposure of UV-B irradiated plants to photo-reactivating light showing that photo-reactivation of UV-B damage is not defective in *uvhl* plants. It was suggested that *uvhl* is defective in a repair or tolerance mechanism that normally provides plants with resistance to several types of DNA damage.

In addition to hypersensitive mutants, phytochrome mutants like the *lh* mutant of cucumber (López-Juez et al., 1992) was used to study the role of UV-B radiation in hypocotyl growth (Ballare et al., 1991). An analysis of different cultivars of maize (Beggs and Wellman 1985), and rice (Reddy et al., 1994) has demonstrated the existence of genetic variability with respect to anthocyanin synthesis in response to UV-B. This shows that suitable genotypes may be found among cultivars or biotypes in the case of natural population to facilitate the studies on UV-B photoreceptor and its responses.

2.3 Photomorphogenic mutants of tomato

2.3.1 Why tomato?

Of all the photomorphogenic mutants available, tomato mutants have been used in the present studies, because of the several features which make it suitable for molecular physiological analysis (table 2.2). Tomato is a widely studied crop species of economic importance with many research groups engaged in developing molecular-genetic techniques for its improvement. It has a relatively small genome, is diploid with twelve chromosomes (n) and is self pollinating. Tomato offers an alternative to *Arabidopsis* as a model system, because of one notable advantage, i.e., individual plants produce a large number (around 2000) of relatively large seeds. Further, tomato seedlings are of large size suitable for physiological and biochemical studies. In addition, there is a large collection of mutants available, among which a few photomorphogenic mutants already exist although they were not initially recognized as such. Tomato is also amenable to *Agrobacterium*-mediated transformation.

Table 2.2: *The comparison of tomato and Arabidopsis as model plants for molecular-genetic analysis*

	Arabidopsis	Tomato
Haploid Chromosome number	5	12
Haploid genome (kB)	7×10^4	7.1×10^5
Gene map available	+	+
Generation time (months)	2	6
Transformation possible	+	+
Tagging possible	+	+
Seeds and seedlings	Small	Large

2.3.2 Phytochrome genes in tomato

Apart from *Arabidopsis*, tomato and sorghum are the only species for which a concerted effort to identify the entire *PHY* family has been reported. The polymerase chain reaction (PCR) derived gene fragments for five tomato *PHY* have been sequenced and compared to the five *Arabidopsis PHY* (Cordonnier-Pratt et al., 1994). These comparisons identified in tomato one *PHYA*, two *PHYB (PHYB* and *PHYB2*), and one each putative *PHYE* and a *PHYF* gene sequences, the latter being so named because it has no special sequence similarity to any known *PHY*. Of the two PHYB genes, neither can be considered an ortholog of *Arabidopsis* PHYB (Pratt et al., In press). Northern analysis has established that each of the tomato *PHY* is expressed as an mRNA sufficiently large (3.8-4.7 kB) to encode a full-length PHY (Pratt 1995). Low stringency genomic southern blots prepared with DNA digested with five endonucleases and probed with fragments of tomato *PHYA, PHYB1, PHYB2, PHYE*, and *PHYF,* and *of Arabidopsis PHYC,* indicated that there are at least four and as many as eight, additional *PHY* in tomato, one of which is probably PHYC. Only when specific DNA probes and antibodies for each member of the gene family are available it will be possible to fully characterize the molecular nature of the mutants discussed below.

2.3.3 Survey of tomato mutants

2.3.3.1 Aurea (au) mutant

The tomato *au* mutant represents one of the most extensively studied phytochrome mutants to date. Mutants of this locus were recognized as photomorphogenic after a new mutant was selected as a non-germinating seed in a screen for gibberellin-deficient mutants (Koornneef et al., 1985). After rescue with gibberellin it was shown to have a long hypocotyl in WL and found to be allelic with previously described *au* mutants (Koornneef et al., 1985) located on chromosome 1 (Khush and Rick 1968).

Adult light-grown *au* plants have yellow-green leaves which are slightly juvenile in appearance, but despite their reduced chlorophyll content, grow extremely well and respond normally to end-of-day-FR (EODFR) treatments (Adamse et al., 1988b) and changes in the R:FR photon ratio (Whitelam and Smith 1991; Kerckhoffs et al., 1992). At the seedling stage, compared to WT the *au* mutant is characterized by a reduction in

(i) hypocotyl growth inhibition in WL, FR,R,B and UV-A (Koornneef et al., 1985; Adamse et al., 1988)

(ii) chlorophyll and Chloroplast development (Koornneef et al., 1985; Ken-Dror and Horwitz 1990; Neuhaus et al., 1993), appearing to lack the VLFR component in the fluence-response curve for greening,

(iii) anthocyanin content (Adamse et al., 1989),

(iv) the photoregulation of the transcript levels of chlorophyll a/b-binding proteins of photosystem-I and II, plastocyanin and subunit II of photosystem I (Sharrock et al., 1988; Oelmüller and Kendrick 1991).

This pleiotropic phenotype, coupled with a lack of phytochrome in etiolated *au* mutant tissues is precisely that predicted for a phytochrome deficient mutant.

Another aspect of the *au* phenotype is its reduced germination in darkness compared to WT (Koornneef et al., 1985). The freshly harvested seeds which are dormant, can be induced to germinate after treatment with a combination of chilling and nitrate (Georghiou and Kendrick 1991) Moreover, exposure to continuous R, an effect which could be replaced by R pulses, led to an increase in germination of *au* mutant seed batches. No inhibitory effect of continuous FR was observed in older seed batches with appreciable dark germination, in contrast to WT which exhibits a strong FR inhibition of germination (Koornneef et al., 1985). Lipucci di Paola et al., (1988) have found a promotion of seed germination by FR for *au* mutants, and suggested that this is a consequence of the absence of an inhibitory FR-HIR.

Etiolated seedlings of the *au* mutant lack spectrophotometrically detectable phytochrome (Koornneef et al., 1985) but in WL-grown mature tissues, contain about 60% of phytochrome compared to WT (Adamse et al., 1988a). Spectrophotometrically active phytochrome has been extracted from WL-grown plants (López-Juez et al., 1990) and has been shown to be recognized by an antibody raised against a fragment of tobacco PHYB (Sharma et al., 1993). Initial studies using antibodies raised against phyA from monocot (Parks et al., 1987; Oelmüller et al., 1989) indicated a lack *of PHYA* in etiolated seedlings. It was also shown that phytochrome mRNA is produced to a similar extent in *au* as in WT

and that this mRNA is functional in an in vitro translation system, and yet, *in vivo*, the protein fails to accumulate (Parks et al., 1987; Sharrock et al., 1988). But, Sharma et al., (1993) using an antibody raised against dicot phyA showed that there was about 20% of the WT level of PHY A present which was stable in R, indicating that it might not be converted to Pfr and is not degraded by the destruction. In addition, one of the tomato phytochrome coding sequence (Sharrock et al., 1988) and a PhyA deficient locus of tomato known as *fri* (Van Tuinen et al., 1994) has been mapped to chromosome 10 whereas the *au* locus is situated on chromosome 1. this means that the deficiency of phytochrome in *au* is at least not due to mutation in the phytochrome A structural gene.

The experiments with *au* line transformed with oat *PHYA3* gene, demonstrated the presence of PHY A3 mRNA in two independent transformants, but in the transformants any PHYA3 apoprotein could not be detected by western blotting (McCormac 1993; Smith et al., 1993). This led to the conclusion that the *au* mutation influenced some process affecting mRNA translation and not chromophore biosynthesis. In a different kind of approach, Kendrick et al.(1994) crossed an oat *PHY A3* overexpressing line (Boylan and Quail 1989) with both the *au* mutant and phenotypically similar *yg-2* mutant, and selected the *au,phyA3* and *yg-2,phyA3* double mutants from the F_2 progeny where both have been shown to accumulate PHYA3 apoprotein. Yet, no rescue of their mutant phenotype was observed, indicating the deficiency of phytochrome chromophore availability. But several attempts to rescue the *au* mutant by feeding chromophore precursors such as biliverdin or phycocyanobilin (Reddy and Sharma, personal communication) have so

far failed to provide direct evidence for the mutation lying in the chromophore pathway, despite the fact that this approach has been successful with chromophore mutants of *Arabidopsis* (Parks and Quail 1991).

2.3.3.1.1 *Aurea* mutant in the elucidation of phytochrome-signaling pathway

One of the variety of biochemical approaches adopted for elucidation of phytochrome-signal transduction is a novel microinjection-based approach to deliver phyA and the putative signaling intermediates directly into the cells of a phytochrome-deficient *au* mutant (Neuhaus et al., 1993; Bowler et al., 1994). Microinjection of purified oat phytochrome A into single cells of the *au* mutant resulted in biochemical complementation of *au* phenotype by exhibiting light-dependent anthocyanin accumulation, and Chloroplast development along with light-dependent expression of a light-responsive *CAB-GUS* reporter construct in the injected cells. The injection of high concentrations of calcium and bovine brain calmodulin was able to stimulate *CAB-GUS* expression and immature Chloroplast development in the absence of phytochrome A. However, anthocyanin accumulation was not affected, suggesting that calcium plays a role in phytochrome A signal-transduction, and that the pathway is branched such that one branch uses calcium and the other does not (Fig. 2.3). In contrast, microinjection of G-protein activators could stimulate full Chloroplast development, and anthocyanin biosynthesis, implicating heterotrimeric-G-proteins as the most upstream component of phytochrome signaling (Neuhaus et al., 1993). Subsequently, Bowler et al.(1994) reported that cyclic-GMP is

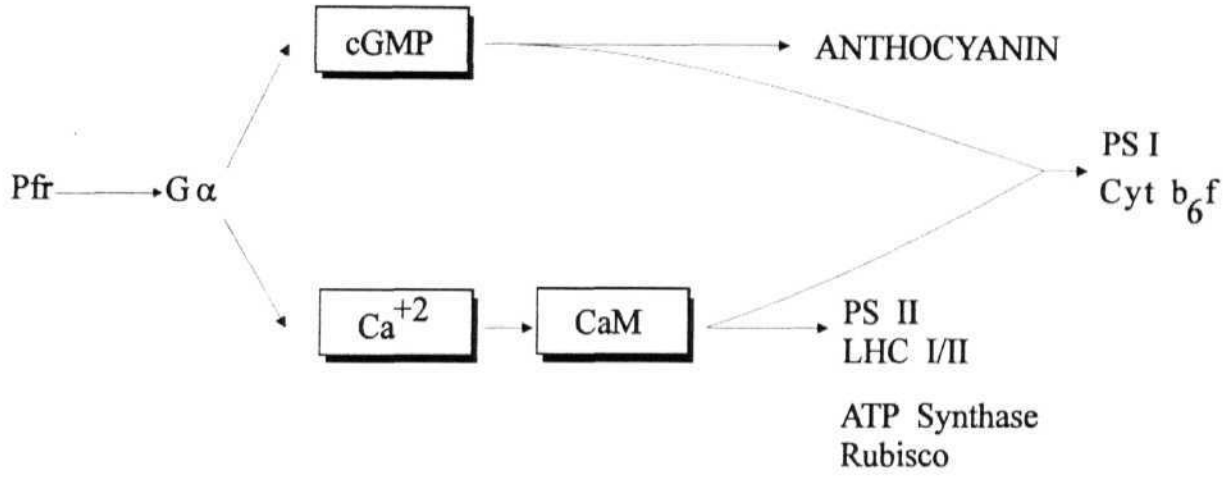


Figure 2.3 Biochemical model of phytochrome signal transduction chain (Adapted from Bowler and Chua, 1994)

able to trigger the production of anthocyanins, and a combination of cGMP and calcium induce the full Chloroplast development containing all photosynthetic machinery (Fig. 2.3). It was also demonstrated that cGMP and calcium act primarily by modulating gene expression.

2.3.3.2 Yellow green (yg) mutants

A recessive mutant which has a phenotype similar, but less extreme than that of the *au* has been called yellow green and has been mapped to chromosome 12 (Koornneef et al., 1985). The *yg-6* mutant has also an *au* phenotype. *yg-2* mutants, like the *au* mutants, are characterized by elongated hypocotyls, reduced germination, and a reduced anthocyanin and chlorophyll content (Koornneef et al., 1985; Buurmeizer et al., 1987). Electron micrographs showed that the number of thylakoid and their degree of stacking in the *au* and the *yg-2* mutant is greatly reduced (Koornneef et al., 1985). The *yg-2* mutant is less extreme than the *au* mutant and older etiolated seedlings have been shown to accumulate some spectrophotometrically detectable phytochrome (Koornneef et al., 1985).

2.3.3.3 Procera (pro) mutant

The phenotype *of pro* mutant is remarkably similar to that of the WT with GA (Jones 1987; Jupe et al., 1988) However, this mutant does not have increased GA levels (Jones 1987). It was suggested by Adamse et al.(1988a) that the *pro* mutant could be similar to the cucumber *lh* mutant, and is a possible candidate for a mutant deficient in the function of the light-stable phytochrome. However, contradicting this possibility, *pro* exhibits an EODFR-elongation response, which is absent in the *lh*

mutant (López-Juez et al., 1990a). The double mutant *au,pro* survives and shows characteristics of both the *au* and *pro* mutant, such as yellow-green leaves of the *au* mutant and a reduction in indentations of the main leaflets and suppression of the development of minor leaflets of the *pro* mutant. In addition, the light-grown *au,pro* double mutant is taller than either the *au* or *pro* mutant.

2.3.3.4 High pigment (hp) mutants

A spontaneous mutant at the *hp* locus was found as early as 1917 (Reynard 1956). Besides this, some other mutants are also similar to the *hp-1* mutant phenotype in some aspects, but map to different loci, such as *hp~2* (Soresi and Salamani 1975), *atroviolatia (arv)* (Rick et al., 1968) and intensive pigment (*Ip*) (Rick 1974).

The monogenic recessive *hp-1* mutants are characterized by features such as dark-green foliage, immature fruit color due to high chlorophyll levels (Sanders et al., 1975), higher lycopene and carotene content resulting in deep-red fruits (Thompson et al., 1962), and high levels of anthocyanin (Kerr 1965; Von Wattstein Knowles 1968). Mochizuki and Komimura (1985) observed that *hp-1* mutant hypocotyls had more anthocyanin than WT when grown in yellow light (YL) and used this as a selection criterion. In addition, Adamse et al.(1989) have selected several new and some extreme *hp-1* mutants (Peters et al., 1989, 1992a). Hypocotyl growth is more inhibited than that of WT when the *hp* seedlings are grown in RL or yellow light (Mochizuki and Komimura 1985), whereas hypocotyl dry weight is lower than in WT when the

seedlings are grown in WL (Von Wettstein Knowles 1968). Thompson et al.(1962) reported that the seed germination of *hp-1* mutants was lower than WT, and that the stems of *hp-1* mutant plants were more brittle resulting in a higher mortality.

The *hp-1* mutant exhibits exaggerated phytochrome responses, whereas the phytochrome content of etiolated seedlings and the physiological characteristics of the phytochrome system are similar to that in WT (Adamse et al., 1989; Peters et al, 1989). Furthermore, plants with *hp-1* like characteristics at their seedling stage were obtained when high levels of the oat *PHYA3* were expressed in tomato (Boylan and Quail 1989). In contrast to WT, the *hp-1* mutant does not require co-action of the blue photoreceptor and phytochrome for normal development. It exhibits maximum anthocyanin synthesis and hypocotyl growth inhibition in RL alone. On the basis of its recessive nature it is proposed that the phytochrome action in etiolated seedlings is under the constraint of the HP-1 gene product (Peters et al., 1992b).

The double mutant of *au* and *hp-1* show no or severely reduced anthocyanin accumulation indicating that at the stage of seedling de-etiolation the *au* mutation is more or less epistatic to *hp-1*. Adult plants of the *hp-1* and the *au,hp-l* double mutant show a quantitatively similar elongation response to reduction in R:FR photon ratio during the daily photoperiod and EODFR treatments (Kerckhoffs et al., 1992; Peters et al., 1992a). However, in WL-grown plants, the *hp-1* mutation appears to have a dwarfing effect in the *au,hp-l* double mutant, particularly so when plants were grown under fluorescent lighting (high R:FR). In addition, it

was noticed that the *hp-1* phenotype is expressed during fruit development in the *au,hp-l* double mutant. These results with WL-grown plants suggest that the *au* mutation is not completely epistatic to *hp-1*, suggesting that the mutation is no longer limiting in mature plants, presumably due to the gradual accumulation of functional phytochromes.

2.3.3.5 Lazy-2 (lz-2) mutant

Of the three gravitropic mutants isolated from tomato, the *lz-1* mutant does not contain sedimenting amyloplasts therefore does not perceive gravity (Roberts 1984); It exhibits agravitropism. The *diageotropica* mutant was suggested to be an auxin-receptor mutant (Kelly and Bradford 1986; Hicks et al., 1989). In contrast, the *lz-2* mutant exhibits a phytochrome-mediated aberrant gravitropic response (Roberts 1987).

Hypocotyls of *lz-2* mutants have a normal gravitropic response when grown in the dark, but exposure to light abolishes its response to gravity and the plant grows downward. At the same time, the ability of the roots to respond to gravity is unchanged by light. Further investigation revealed that RL causes the downward growth, whereas treatment with BL did not alter the dark-grown WT gravity response. *lz-2* seedlings irradiated with FRL pulse immediately after RL pulse exhibited no downward growth. However, continuous R or FRL both resulted in downward growth of *lz-2* seedlings, indicating the involvement of phytochrome in this response (Gaiser and Lomax 1993). Both dark and light-grown *lz-2* plants are capable of generating a normal growth asymmetry as the phototropic response of these plants is identical

to WT. Also, *lz-2* hypocotyls are capable of responding to exogenously applied IAA in a nearly WT fashion (Gaiser and Lomax 1992). Taken together, it was suggested that *lz-2* mutation is in a gene that affects some step subsequent to the gravity perception and gravitropic signal transduction (Gaiser and Lomax 1992), and perhaps involves an intersection point between the light and gravity response mechanisms.

2.3.3.6 Type specific phytochrome mutants of tomato

2.3.3.6.1 Far-red insensitive *(fri)* mutants

In search for type specific phytochrome mutants Van Tuinen et al.,(1994) have selected two recessive mutants of tomato with slightly longer hypocotyls than the WT under low fluence rate screens of BL and RL. These two mutants were shown to be allelic and further analysis revealed that hypocotyl growth was totally insensitive to FR light. Consequently, they named the locus *FR insensitive*. *fri* mutants have been shown to lack the bulk pool of phytochrome in etiolated seedlings (predominantly phyA) and immunologically detectable PHYA (Van Tuinen et al., 1995a). A phyB like polypeptide is present in normal amounts and a small stable phytochrome pool can be readily detected by spectrophotometry in *fri* mutants (Van Tuinen et al., 1995a). In addition, northern analysis shows the PHYA mRNA is modified in *the fri* mutants. The *fri* locus has been mapped to chromosome 10, as has the PHYA gene. Young WL-grown *fri* mutant plants are almost indistinguishable from the WT. Adult plants of the *fri* mutants show retarded growth and are prone to wilting, but exhibit a normal elongation response to FR given at the end of the daily photoperiod. Based on the above observation it is proposed that these *fri* mutants are putative

phytochrome A mutants which have normal pools . of other phytochromes.

2.3.3.6.2 Temporarily red light insensitive (*tri*) mutant

Another group of recessive long-hypocotyl mutants were selected under WL or low fluence RL (Kendrick et al., 1994) and were shown to be blind to RL during the first two days after transfer from darkness irrespective of their physiological age, (Van Tuinen et al., 1995b) consequently, they are named *temporarily red light insensitive* mutants. Four alleles have been isolated; when examined by western analysis, one of these had none (below detection limit) and one had a reduced amount of a PHYB-like protein as compared to WT the other two alleles had polypeptide recognized by the PHYB like protein in the WT. *tri* locus was mapped to chromosome #1. The molecular analysis of four *tri* alleles revealed that in these mutants the transcripts of *PHY. A, PHYB2, PHYE,* and *PHYF* were indistinguishable in size and abundance from WT. In contrast, the size of the *PHYB*, transcript was similar to WT but much reduced in abundance in the mutant, indicating that *th* alleles are putative mutants in the structural gene that encodes the PHYB1 apoprotein (Kerckhoffs et al., 1995). The WL grown plants of this mutant are slightly taller than the WT, but otherwise very similar. One interesting feature of *tri* mutants is that, in contrast to the absence of an elongation growth response to far-red light given at the end of the daily photoperiod in all phyB-deficient mutants so far characterized, the *th* mutant responds to EODFR treatment, and also shows a strong response to supplementary day time FR. This suggests that phyB 1 and phyB2 are

closely related or they might both be able to regulate the EODFR response in tomato.

2.4 Conclusions

Isolation of photomorphogenic mutants and their biochemical and genetic analysis is currently the most important approach to understand the role of light in plant development. In *Arabidopsis,* a wide range of mutants which are either defective in photoperception or signal transduction or terminal response have been identified. Considering the rapid pace with which they are being analyzed, very shortly one can expect to have a thorough understanding of every step involved in photomorphogenesis of *Arabidopsis.* Since process of de-etiolation is not same in seedlings of all plant species, a comparative study with different plant species is very essential. The availability of a good number of photomorphogenic mutants (other than *Arabidopsis)* in tomato makes it a very valuable complement system for conducting comparative studies with *Arabidopsis.*

Already the physiological studies of these mutants and their isogenic WT have provided a good indication about the roles of phytochrome A and B, as well as about roles for other phytochromes. In *Arabidopsis* seedlings, the absence of phyB leads to excessive elongation of hypocotyl, stem, petioles, root hairs, and acceleration of flowering, indicating that phyB plays a major role in regulating growth and development. In contrast to this, phyA appears to play only a minor role in photomorphogenesis; though phyA is involved in the photocontrol of

cell elongation, phyA mutants are virtually indistinguishable from WT plant when grown in WL. In contrast, phyB mutants display reduced cotyledon expansion and chlorophyll accumulation. The absence of phyA does not appear to significantly affect the de-etiolation process. Furthermore, it was showed that in addition to Pfr, form the Pr form of phyB has an activity, promoting negative gravitropism and inhibiting germination.

From the earlier studies we can expect that the availability of mutants that are doubly null for phytochrome A and B will reveal more about the roles of these and the other phytochromes, and also allow the design of mutant-screening strategies for the identification of mutations affecting other phytochrome species. Analysis of blue light mutants resulted in identification of two loci *hy4* and *nph1* encoding putative BL receptors of which one is for the BL-mediated hypocotyl elongation and the other for phototropism.

A brief overview of recent advances in the understanding of plant signal-transduction mechanism will illustrate how indispensable the mutants are in these studies. Genetic analysis of mutants in *Arabidopsis* led to the identification of novel proteins such as DET1, COP1, etc., in phytochrome signaling and it was also implicated that these proteins are not only specific for phytochrome signaling but are part of negatively acting global regulators for plant development that are able to receive signals from multiple stimuli and integrate them into developmental responses. In contrast, the biochemical approach has identified the G-proteins, cyclic nucleotides, Ca-calmodulin mediated signaling systems

in plant signal-transduction, which were earlier thought to be specific for animal systems. Both the genetic and biochemical approaches clearly reveal the details of a variety of signaling networks. A combination of these approaches will allow both the elucidation of mechanisms and the identification of the molecules responsible, which will finally lead to the thorough understanding of how plants perceive, respond, and adapt to changing developmental and environmental stimuli.

3. MATERIALS AND METHODS

3.1 Plant material

Four genotypes of tomato {*Lycopersicon esculentum* Mill. cv Ailsa Craig) the *au* mutant, the *hp* mutant, the *au,hp* double mutant, and their isogenic wild type were used in this study. Tomato seeds used in this study were harvested in 1989, 1990, and 1991, and were obtained from Dr. R. E. Kendrick. In addition, seeds multiplied at School of Life Sciences, University of Hyderabad, (1994) from the stock obtained from Wageningen, The Netherlands were also used in this study.

The *au* mutant was isolated as a long hypocotyl mutant by Koornneef et al., (1981, 1985) and has been identified as a photoreceptor deficient mutant which lacks the spectrally detectable phytochrome in etiolated seedlings; and the mature plants are characterized by yellow green foliage.

The *hp* mutant was isolated as a spontaneous, monogenic recessive mutant as early as in 1917. It is characterized by mature fruits that have a higher lycopene and carotene content, darker foliage and immature fruit color than that of the wild type and possess equal amounts of phytochrome to that of the wild type.

The *au,hp* double mutant was isolated from crossings between these *au* and *hp* mutants (Adamse et al., 1989). The overall morphology and phytochrome content of *au,hp* resembled the *au* phenotype.

3.2 Seed storage and seedling growth

Seeds were stored in polythene bags along with a desiccant at 4°C till use; seeds were sown after sterlizing with 0.1% (v/v) Na-hypochlorite solution) for 10 min. Thereafter, seeds were washed with DW and sown in transparent plastic boxes (9.5 cm / x 9.5 cm *b* x 5 cm *h*) (Boxes were also surface sterilized with 0.1% Na hypochlorite solution (v/v)) containing 20 ml of 0.5% (w/v) agar support prepared either with 1/10th of concentration of MS medium (Inorganic salts only; Murashige and Skoog 1962) or 5 mM potassium nitrate. MS medium was used for the experiments of PAL induction and anthocyanin accumulation and potassium nitrate was used for all the other experiments. Unless otherwise specified, seedlings were grown at 25±1°C in absolute darkness for 96 h and then transferred to continuous RL or WL. In the case of BL pretreatment, 84 h old dark-grown seedlings were used.

3.3 Light sources

The red light source consisted of two 47 inches long cool-white fluorescent tube lights (40 W x 2) whose output was filtered through two layers of 6 mm thick red plexiglass sheets (λ_{max} 650 nm) (Manga 1987). The ends of the tube lights were wrapped in black paper to prevent far-red light leakage. The distance between plants and light source was about 55 cm and the intensity of RL at the top of the plants was 0.60 Wm^{-2}.

Long wavelength FR light was obtained by filtering the output of a 300 W projector through Schott RG-9 interference filters (λ_{max} 756 nm), FR light intensity at the top of the seedlings was 6.0 Wm^{-2}. Blue light (λ_{max} 450 nm) was obtained using a setup similar to obtaining RL except that red plexiglass sheets were replaced by blue plexiglass sheets (λ_{max} 450 nm). Blue light intensity was 0.13 Wm-2 at the top of seedlings. For dark controls, after sowing of seeds, the plastic boxes were wrapped in black cloth and were kept in a black cardboard box at 25±1 °C.

All manipulations of seedlings were done under a dim-green safe light, obtained by filtering the output of a cool-white fluorescent tube light (40 W) through six layers of green cellophane paper (λ_{max} 530 nm). The ends of the tube lights were covered with black paper. The intensity of the green light was not greater than 0.01 W m^{-2} and light exposure did not last longer than 2 min at any given time.

3.4 Anthocyanin estimation

Ten seedlings of uniform height were harvested and were dissected into cotyledons and hypocotyls. Both organs were extracted separately with 1.2 ml of acidified methanol (1% HCl, w/v) for 48 h in darkness at 25°C with constant shaking. A Folch partitioning was performed after adding 0.9 ml of water and 2.4 ml of chloroform to the extracts and centrifugation for 20 min at 1,600 x *g*. The absorbance of the top phase was determined at 535 nm.The anthocyanin amount was expressed as A_{535nm}/10hypocotyls or pairs of cotyledons (Adamse 1988)

3.5 Chlorophyll estimation

Chlorophyll content was determined by using the method of Arnon (1949). Chlorophyll was extracted from cotyledons by homogenizing five pairs in 200 ul of 80% acetone (v/v) and centrifuged at 3000 x g. The chlorophyll content was estimated by taking 12.5 ul of supernatant in 5 ml of 80 % acetone, and the absorbance was read at 652 nm and 710 nm and the concentration was calculated by using (Arnon 1949) the formula: A_{652}-A_{710} x 11.11 = mg Chlorophyll.ml-1,

3.6 Protein estimation

The protein estimations were done according to Lowry et al.(1951). The protein in crude extracts was first precipitated by mixing with an equal amount of 10 % TCA and incubating overnight. The precipitate was separated by centrifugation at 3000 x *g* and redissolved in 0.1 N NaOH to avoid inhibitory compounds in crude extracts (Peterson 1983). Protein was estimated after taking an aliquot from redissolved sample. The standard curve was prepared using the bovine serum fraction V as a standard protein (Fig. 3.0

3.7 Phenylalanine ammonia lyase extraction and assay

Twenty hypocotyls or pairs of cotyledons were homogenized at 4°C in a pestle and mortar with 0.2 g of sea sand and 0.15 g of polyvinylpolypyrrolidone in 3 ml 0.1 m borate buffer (pH 8.8) containing 54 mM mercaptoethanol. The homogenate was centrifuged at 18,200 x *g* for 30 min at 4°C and supernatant was applied to a Sephadex G-25 column (2 cm diameter x 10 cm *l*) equilibrated with 0.1 M borate buffer (pH 8.8). The fractions constituting void volume were

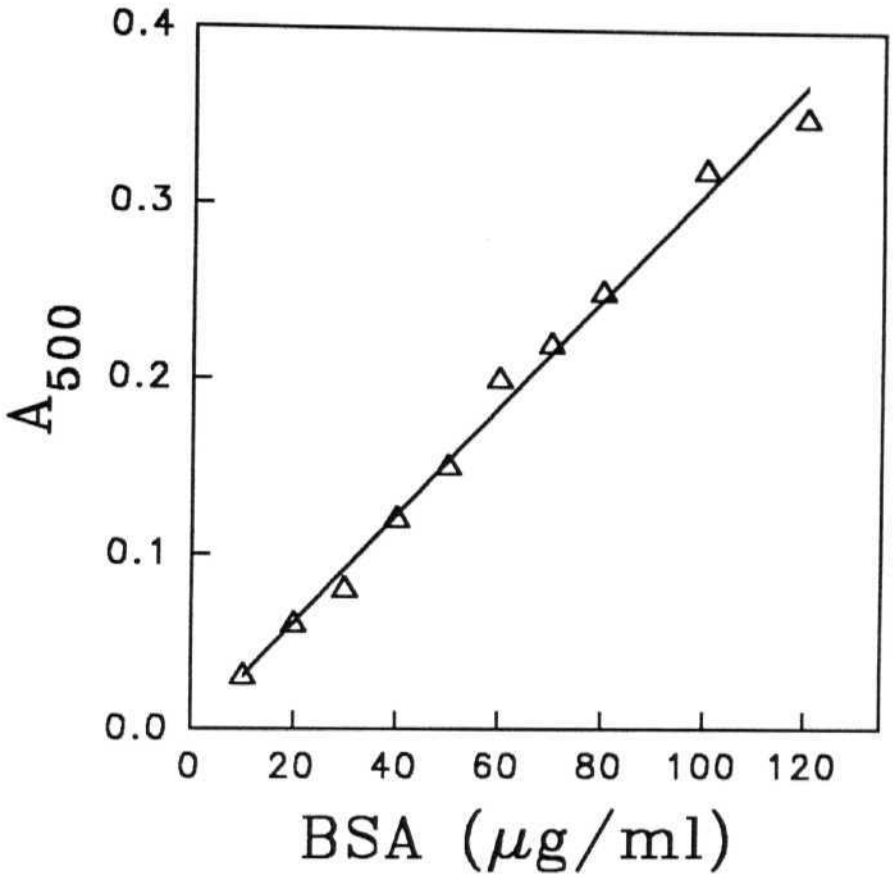


Figure 3.1: Standard curve of protein estimation. Protein was estimated by the method of Lowry et al., (1951) Bovine serum albumin fraction V (BSA) was used as standard protein.

(w/v) sulphanilamide in 3 N HCl and measuring the absorbance at 540 nm (Snell and Snell 1949) using the standard curve (Fig. 3.2).

3.9 Nitrite reductase extraction and assay

Five hypocotyls or pairs of cotyledons were homogenized at 4°C with 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5) in a precooled pestle and mortar on ice; the homogenate was centrifuged at 30,000 x g for 30 min at 4°C and the clear supernatant was used for assay. The NiR (EC 1.7.7.1) assay was performed at 30°C for 1 h in an assay mixture containing 200 ul of extract, 0.04 mM potassium phosphate buffer (pH 7.5), 0.27 mM methyl viologen, 0.5 mM KNO_2 and 2.5 mM sodium dithionate in a final volume of 0.5 ml (Vega et al., 1980). The reaction was terminated by vigorous vortexing, till the blue color disappeared and the amount of nitrite utilized was estimated as described under NR extraction and assay (Fig. 3.2).

3.10 Dose response curves for NR and NiR

The concentration of nitrate to be maintained in the growth medium of tomato seedlings for photoinduction of NR and NiR activities was determined by growing the seedlings on different concentrations of potassium nitrate ranging from 5 to 60 mM. Both NR (Fig. 3.3) and NiR (Fig. 3.4) activities were assayed as described above. The dose response curve was constructed by plotting the enzyme activity against different concentrations of nitrate. The induction of NR and NiR showed a biphasic curve with the first phase being saturated at 10 mM nitrate and

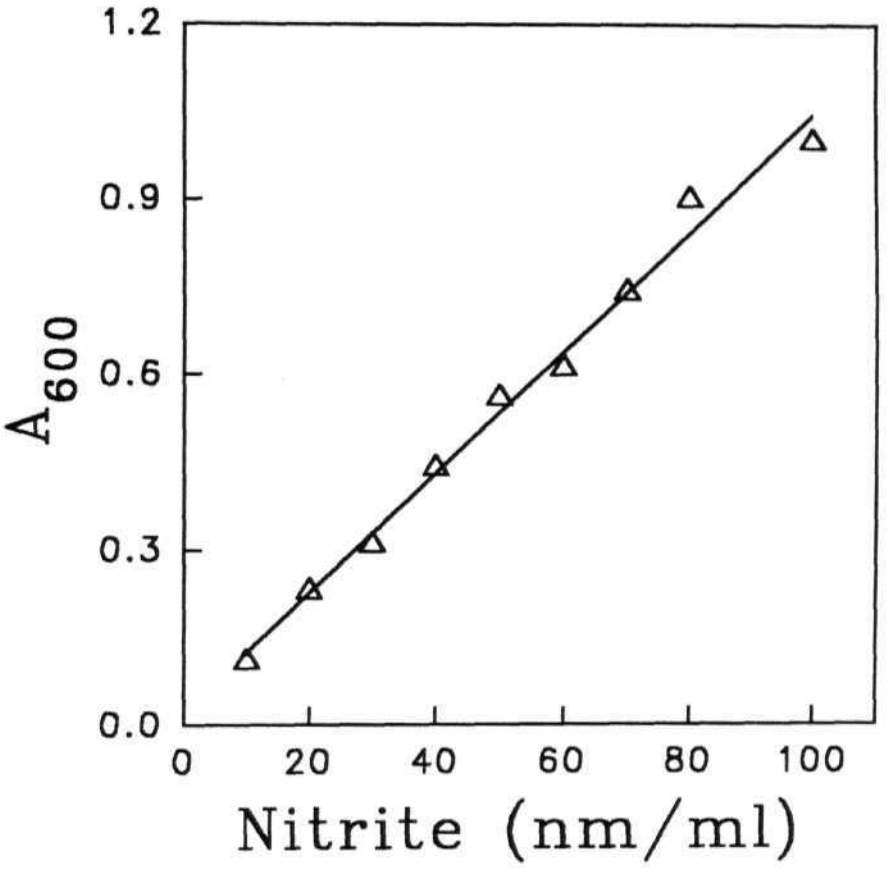


Figure 3.2: Standard curve of nitrite estimation. Nitrite was estimated by the method of Snell and Snell (1949).

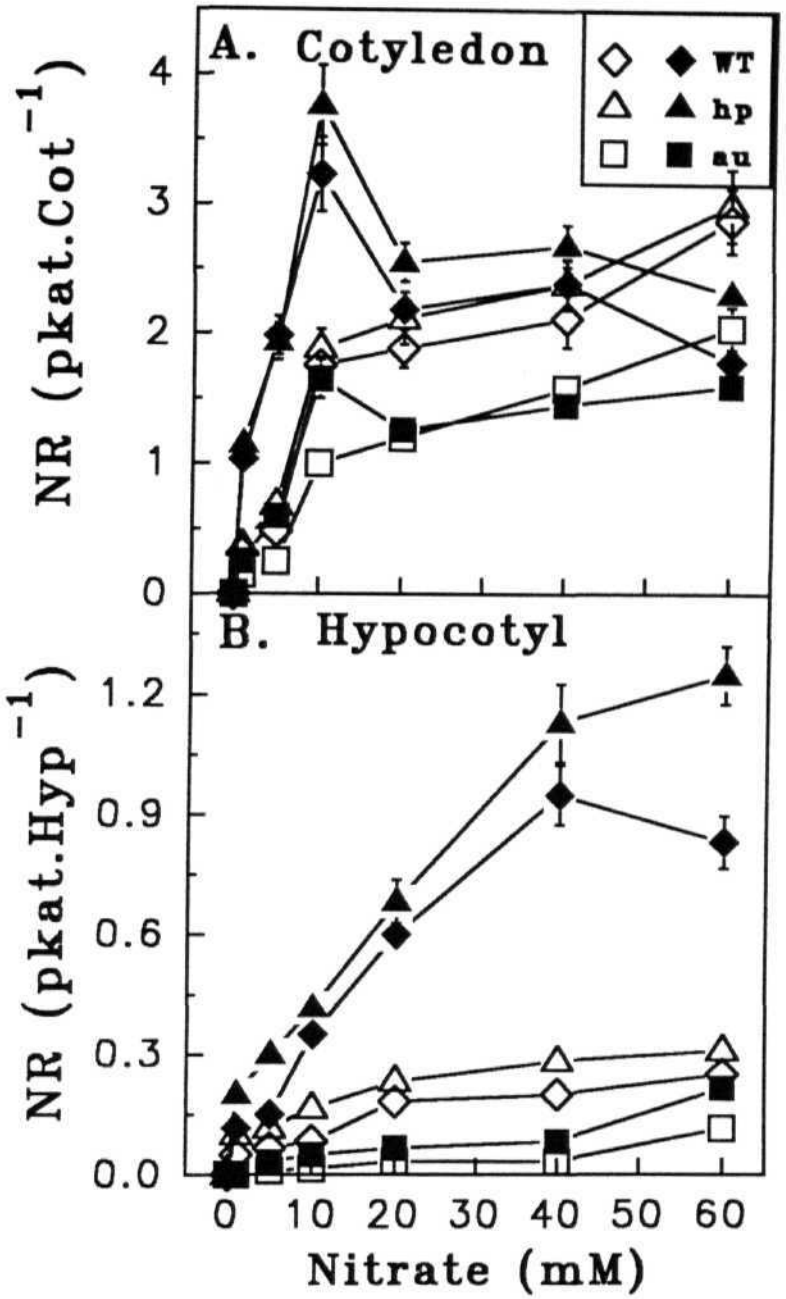


Figure 3.3: Dose response curve of NR in cotyledons (A) and hypocotyl (B) of tomato seedlings. Seedlings were grown on agar support medium containing different concentrations of nitrate. Seedlings were grown upto 96 h from sowing in darkness, and were then transferred to continuous RL (closed symbols). The control seedlings were maintained in darkness (open symbols). NR activity was measured after 120 h from sowing.

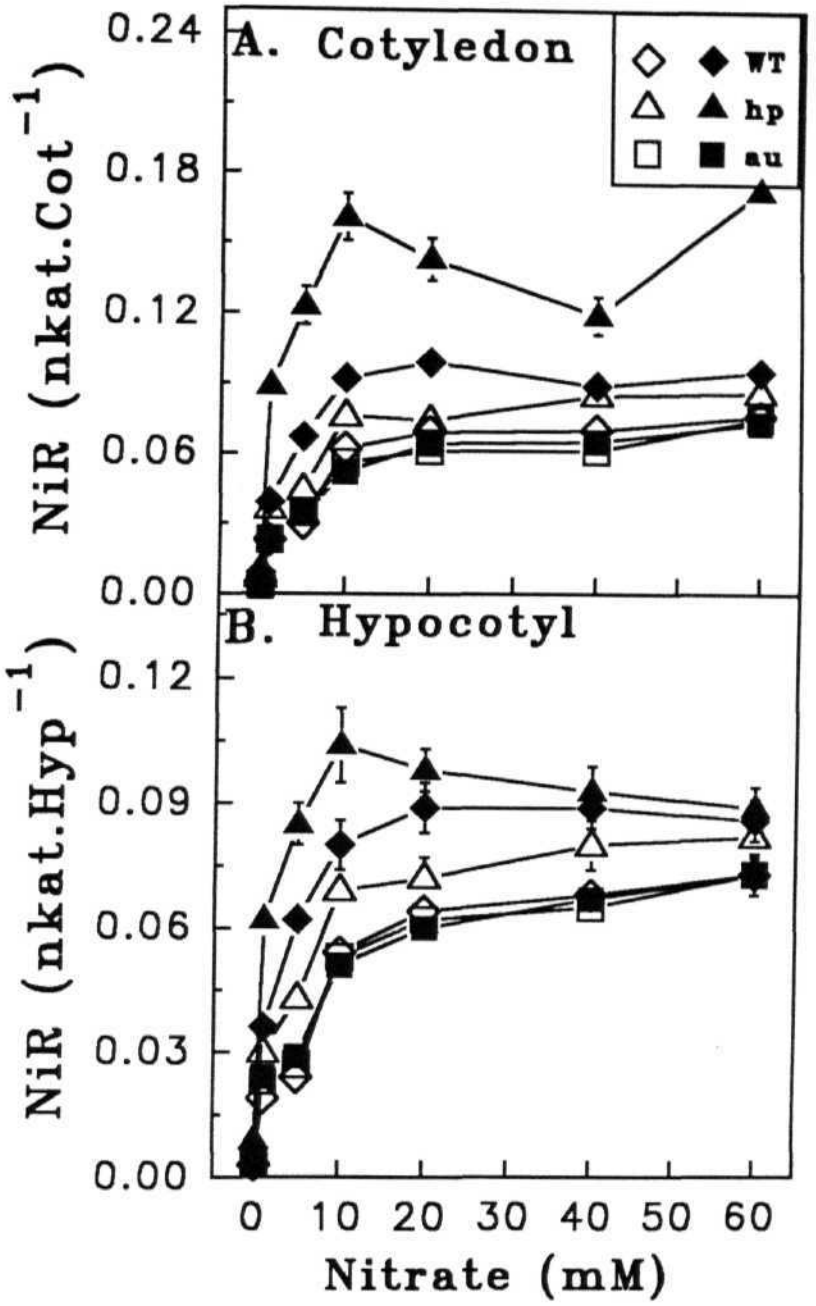


Figure 3.4: Dose response curve of NiR in cotyledons (A) and hypocotyl (B) of tomato seedlings. Seedlings were grown on agar support medium containing different concentrations of nitrate. Seedlings were grown upto 96 h from sowing in darkness, and were then transferred to continuous RL (closed symbols). The control seedlings were maintained in darkness (open symbols). NiR activity was measured after 120 h from sowing.

the second phase continued upto 60 mM nitrate concentration. In view of this, we used 5 mM concentration of nitrate for all subsequent experiments which represents the mid-point for substrate-mediated induction of enzyme activity for the first phase.

3.11 Amylase extraction and assay

Twenty hypocotyls or pair cotyledons were homogenized in chilled pestle and mortar on ice in 3 ml of buffer containing 0.1 M sodium acetate (pH 5), 4 mM $CaCl_2$. The homogenate was centrifuged at 30,000 x g for 20 min at 4°C and clear supernatant was used for assay. The amylase assay was performed in the reaction medium containing 100 mM sodium acetate (pH 5.2) 4 mg/ml amylose, 4 mM $CaCl_2$, 1 mM sodium fluoride and 500 µl of supernatant in a final volume of 4 ml (Vally and Sharma 1991) at room temperature. The reaction was conducted for 2 h, withdrawing 500 µl aliquotes at 1 h intervals. The amount of reducing sugars in the aliquots was estimated by adding an equal volume of dinitrosalicylic acid reagent (Bernfeld 1955); after mixing, the samples were boiled for 5 min and then were diluted to 3 ml with distilled water. The increase in reducing sugars was determined by measuring absorbance at 540 nm using maltose as standard (fig 3.5)

3.12 Inhibitors

a-aminoxy, β-phenylpropionic acid (0.4mM), cycloheximide (25 µg ml^{-1}), cordycepin (200 µg ml^{-1}), puromycin (200 ug ml^{-1}), actinomycin-D (100 ug mH), norflurazon (0.06 mM) and sodium tungstate (2 mM) were used in different experiments. In the experiments

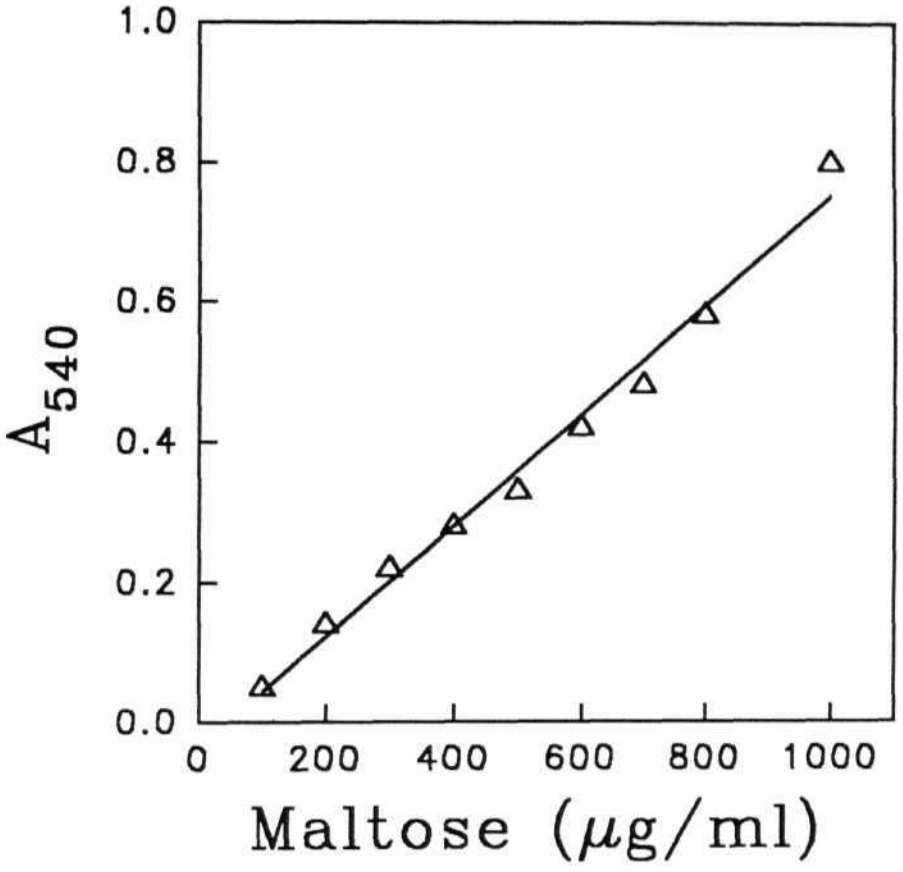


Figure 3.5: Standard curve of maltose estimation. Maltose was estimated by the method of Bernfeld (1955).

where inhibitors were applied from the time of sowing, inhibitors were mixed with agar before solidification. In other experiments, required amounts of inhibitors were sprayed on the seedlings under green safe light 2 h prior to onset of light irradiation.

3.13 Sample preparation for SDS-PAGE

Ten pairs of cotyledons or hypocotyls were homogenized in a mortar and pestle mounted on ice in 500 µl of extraction buffer containing 50% ethylene glycol, 100 mM Tris HCl pH 8.3, 140 mM NH_4SO_4, 10 mM EDTA and 20 mM Na-bisulphite, centrifuged at 10,000 x g for 20 min. Supernatant was mixed with 1/5th volume of 5X sample buffer containing final concentrations of 62.5 mM Tris-HCl pH 6.8 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) ß-mercaptoethanol and 0.005% bromophenol blue (w/v). The samples were boiled for 5 min at 100°C for uniform coating of detergent, on a boiling water bath. After cooling, samples were again centrifuged at 10,000 x *g* for 10 min to clarify the sample from any undissolved material and the clear supernatant was used for loading on SDS-PAGE gels.

3.14 SDS-PAGE

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

8% (w/v) Acryiamidc was polymerized as 1 mm thick separating gel (Size: 9 cm / X 6.5 cm *b)* in 0.375 M Tris-HCl buffer pH 8.8 containing 0.1% (w/v) SDS,0.05% (w/v) ammonium persulphate and

0.03% (w/v) TEMED. 4% stacking gel (w/v) was made in 0.125 M Tris-HCl pH 6.8, containing 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.03% (w/v) TEMED. The tank buffer for electrophoresis consisted of 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1% SDS (w/v). After the polymerization of the stacking gel is complete, the sample wells are rinsed with DW to remove any unpolymerized substances. The samples prepared as described in section 3.13 were loaded into the bottom of the well using a Hamilton microlitre syringe and unused wells were filled with 1X sample buffer. The electrophoresis was carried out at room temperature for 2 h and 30 min at a constant voltage of 120 V. The following marker proteins were used: Phosphorylase-B (MW 97,000), Bovine serum albumin (MW 66,000), and egg albumin (MW 45,000). all the marker proteins were dissolved at 1 mg/ml concentration in the sample buffer and 5 µl mixture was loaded on SDS-PAGE gel.

3.15 Western blotting

Antigens were separated by SDS-PAGE as described above. The gel was soaked in transfer buffer (pH 8.3) containing 39 mM glycine, 48 mM Tris base, 0.037% SDS (w/v) and 20% methanol (v/v) for 15 min. The proteins were transferred electrophoretically (Burnette 1981; Towbin et al., 1979) on to a PVDF (Immobilon-P from Millipore) membrane. The PVDF membrane was first wetted in methanol for 1 min and then soaked in transfer buffer before use. The gel was sandwiched with the membrane and three layers of Whatmann 3MM paper pre-soaked in transfer buffer was layered on both sides of sandwich . The electrophoretic transfer was performed using a custom built dry blot

apparatus made of two graphite plate electrodes (10 cm / x 7.5 cm *b*), applying a constant current of 0.65 $mA.cm^{-2}$ at room temperature for 2.5 h.

After completing the transfer, the non-specific protein binding sites on the membrane were blocked by incubating the membrane in 10 ml of blocking buffer for 1 h. The blocking buffer consisted of 2°/P nonfat dry milk (Johnsen et al., 1984) prepared in TBST (Tris buffered saline + Tween 20) buffer (pH 8.0) containing 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20. After washing in 10 ml of TBST for 5 min, the membrane was incubated with 8 ml of tobacco PAL antibody raised in rabbit (1:350 dilution in TBST) for 1 h with gentle shaking, and then washed three times for 10 min each with 10 ml TBST to remove the unbound primary antibody from the membrane. It was then incubated for 1 h with secondary antibody which is an alkaline phosphatase coupled anti-rabbit IgG (1:7,500 dilution in TBST) obtained from Sigma chemical company, USA. It was again washed thrice for 10 min each with 10 ml of TBST to remove unbound secondary antibody.

Antigen antibody complexes on the membrane were visualized by incubating it in a chromogenic mixture prepared by adding 33 µl of BCPIP (5-bromo-4-chloro-indolyl phosphate; 50 $mg.ml^{-1}$) and 66 µl of NBT (Nitro blue tetrazolium; 50 $mg.ml^{-1}$) to 10 ml of alkaline phosphatase buffer (pH 9.5) containing 100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris-HCl. The reactive areas became purple, and when intensity of color developed to the desired level, the reaction was stopped by

rinsing the membrane in deionized water for a few minutes, changing the water at least once.

3.16 Monitoring of carbon dioxide dependent oxygen evolution

Carbon dioxide dependent oxygen evolution in intact cotyledons was monitored by using a Clark type oxygen electrode (model DW2, Hansatech, Kings Lynn, UK). The sample chamber of the monitor was filled with equal parts of carbon dioxide buffer containing 7 parts of 0.1 M $NaHCO_3$ + 3 parts of 0.1 M Na_2CO_3 and Avrons medium containing 15 mM Tris-HCl pH 7.8, 2 mM NaCl, 4mM $MgCl_2$ and 4 mM K-phosphate (Sharma et al., 1979). The oxygen evolution was monitored by using three pairs of cotyledons chopped into two pieces each were used as sample. A constant temperature of 30°C was maintained during the course of the experiment by circulating water through the outer jacket of the reaction chamber from a constant temperature refrigerated circulating water-bath. The electrode was calibrated with air-saturated distilled water. The samples were first incubated in the dark to establish the respiration rate, and then the oxygen evolution was monitored by exposing the samples to light. A constant rate of oxygen uptake due to respiration was established as the basal rate before irradiation from which the rate of oxygen evolution was calculated. Light source consisted of a 35 mm slide projector (Atul Electronics Corporation, India. Lamp: Xenophat (halogen) 24v/150W) whose intensity is 1250 $\mu E\ m^{-2}S^{-1}$.

3.17 Scanning electron microscopy

Seedlings grown under 24 h continuous red light after 96 h dark and 120 h continuous dark were used. 1 cm portion below the hook region was cut and mounted on 1 cm diameter sample holding stub with the help of a small hook. The stub along with the sample was immersed in liquid nitrogen for about two minutes and immediately observed and photographed under Jeol JSM-35 scanning electron microscope at an accelerating voltage of 25 V and magnification 1 10x.

4. RESULTS

Tomato photomorphogenic mutants have nearly identical phenotypes of seedlings when grown in darkness (Fig. 4.2A) except that the *au* seeds show a reduced and delayed germination. In the present study on an agar medium supplemented with inorganic salts of MS media, the *au* and WT seeds germinated with high frequency. The fact that these mutants respond differently to light is clearly apparent from the phenotype of young and also of light-grown plants of *au* and *hp*, which is very distinct from the WT plants (Fig. 4.1). *au* plants have yellow green leaves which are slightly juvenile in appearance, whereas *hp* have a dark-green foliage; the *au,hp* double mutant is very similar to the *au* mutant. We studied photophysiological responses in WT and mutants with respect to a few morphological markers, enzyme induction, and Chloroplast development.

4.1 Morphological features of seedlings

In general, WT, *au,* and *hp* seedlings grown in continuous darkness were morphologically similar (Fig. 4.2A). In comparison, seedlings grown under WL responded differently with respect to induction of photomorphogenesis. While the WT and *hp* seedlings were short and green, *au* and *au,hp* mutant retained largely an etiolated phenotype by having an elongated hypocotyl, and reduced greening. However, mutants and WT seedlings showed the light-triggered hypocotyl hook opening, and also cotyledon expansion, indicating that

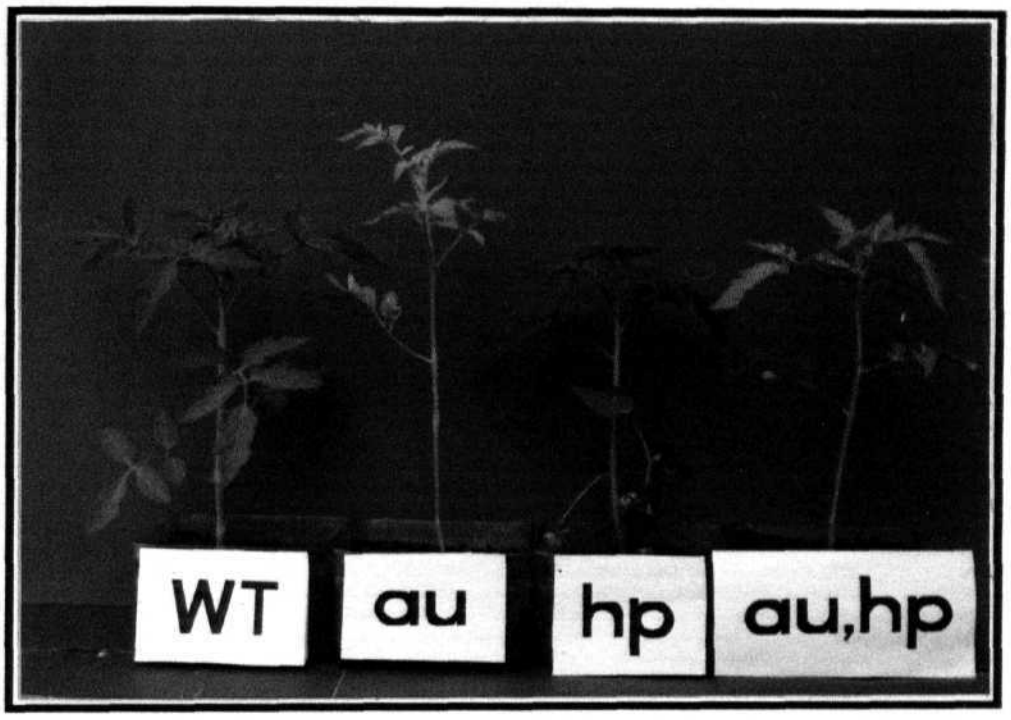


Figure 4.1: The phenotype of the wild-type and photomorphogenic mutants of tomato. Six week old field grown plants of the wild-type (WT), *aurea (au)*, high-pigment *(hp)*, and *au,hp* mutant plants. Note the yellow appearance of the *au* mutant and *au,hp* double mutant is more like *au* mutant than *hp*.

Figure 4.1: The phenotype of the wild-type and photomorphogenic mutants of tomato. Six week old field grown plants of the wild-type (WT), *aurea (au)*, high-pigment *(hp),* and *au,hp* mutant plants. Note the yellow appearance of the *au* mutant and *au,hp* double mutant is more like *au* mutant than *hp.*

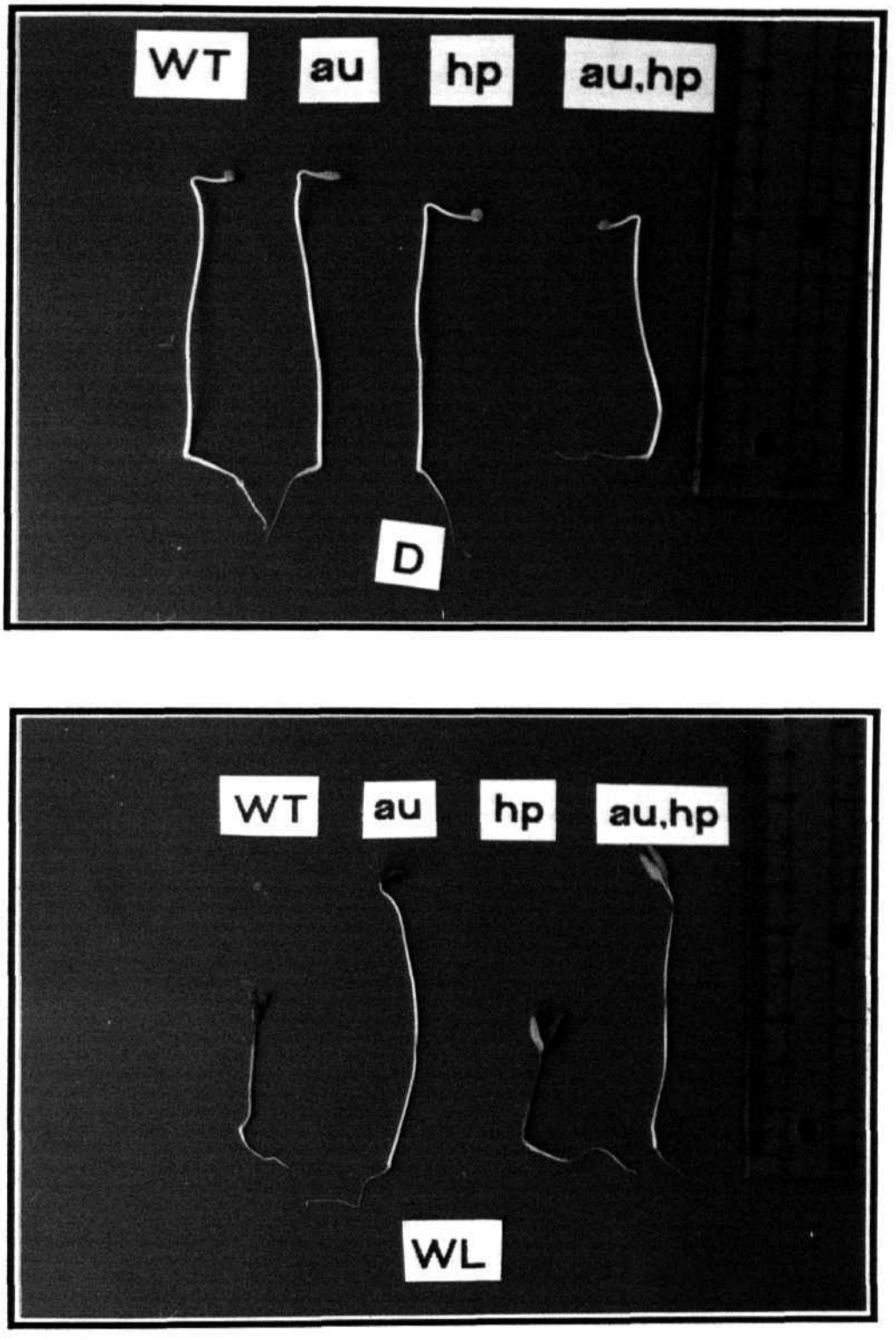


Figure 4.2: The phenotype of dark and white light grown seedlings of wild-type and photomorphogenic mutants of tomato. Six day old seedlings of WT, *au*, *hp*, and *au,hp* grown in complete darkness (D). Six day old seedlings of WT, *au*, *hp*, and *au,hp* grown under continuous white light (WL)

these light-triggered responses operate normally in these seedlings (Fig. 4.2B).

4.1.1 Hypocotyl elongation

One of the earliest effects of light on germination is the inhibition of hypocotyl elongation. In seedlings grown under WL, a high degree of light-mediated inhibition of hypocotyl elongation was observed in WT and *hp* seedlings, whereas *au* and *au,hp* failed to respond to it (Fig. 4.2B).

4.1.2 Cotyledon expansion

In dicot seedlings, expansion of cotyledon is initiated only after exposure to light, leading to a parallel increase in size and accumulation of fresh mass. Expansion of cotyledons was monitored by studying the time course of increase in fresh weight of cotyledons under RL and WL (Fig. 4.3). In seedlings transferred to RL, cotyledon fresh mass increased over 3 fold in the next 48 h in WT and *hp*. However, the increase in *au* was only 10% over the respective dark controls. By contrast,, when *au* seedlings were transferred to WL, the accumulation of fresh mass was similar to the WT. In WL, *hp* seedlings showed a rapid gain of mass initially, but by 48 h it was equal to WT. There was absolutely no fresh mass accumulation in cotyledons of dark-grown seedlings during this period in all the three genotypes.

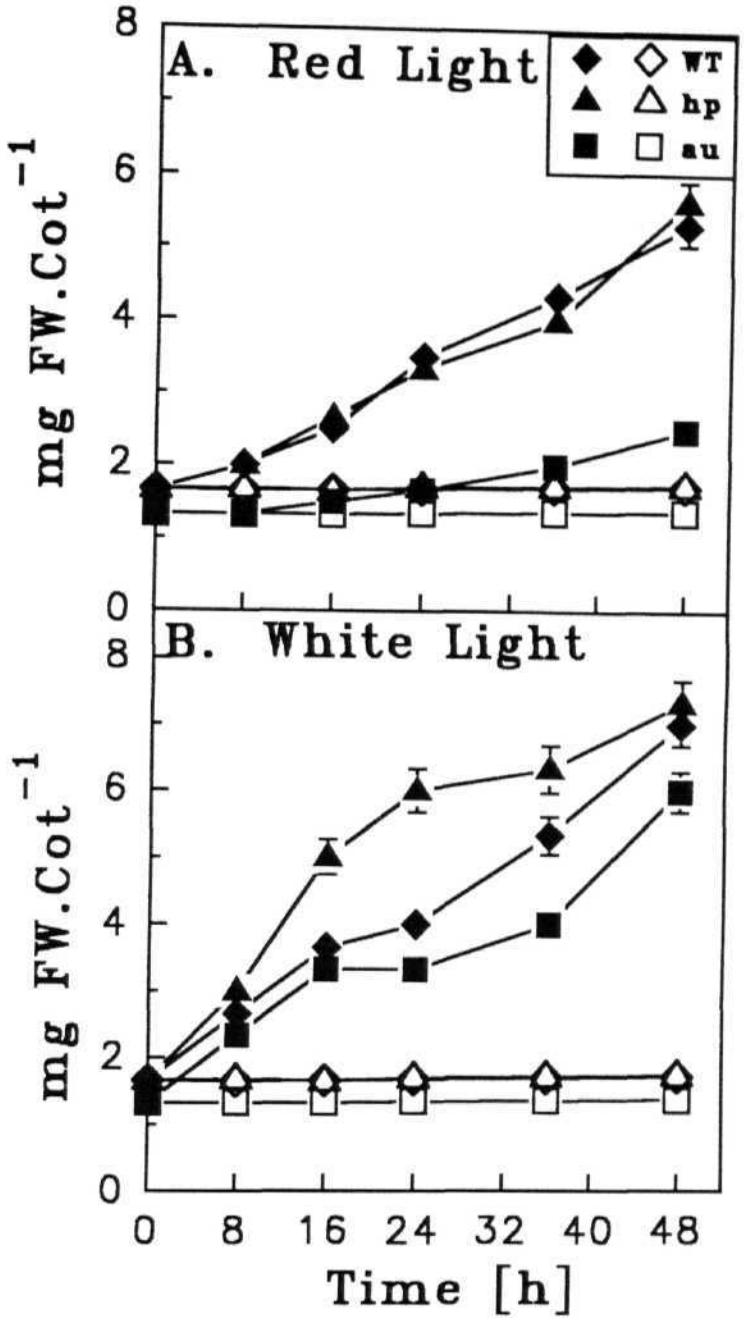


Figure 4.3 Time course of change in fresh weight of cotyledons of tomato seedlings. Seedlings were grown in darkness upto 96 h from sowing and then transferred to red light (A) and white light (B).

4.1.3 Hypocotyl hair

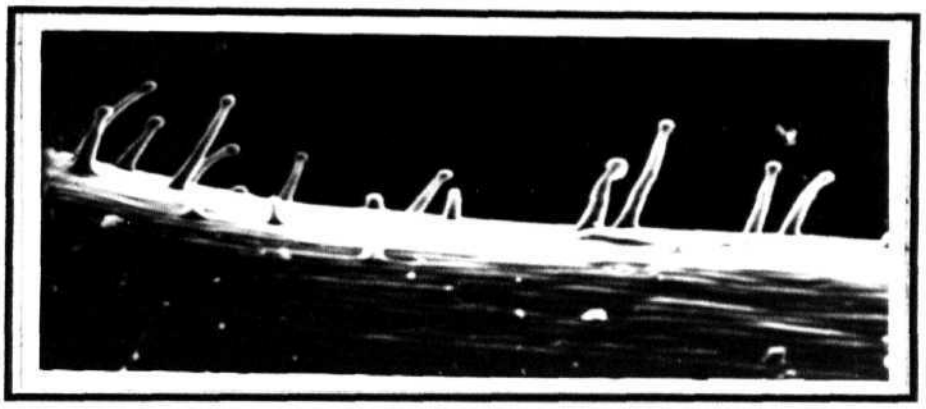
The most important aspect of developmental biology is to know how a cell learns to identify itself and differentiate. The simplest processes to study differentiation would be those that are readily visible like root hair and hypocotyl hair. These are subsets of epidermal cells which undergo several morphological changes before differentiating into hair. In tomato, trichome (hair) density on leaves was reported to be influenced by length of the photoperiod (Gianfagna et al., 1992). To understand better the role of light, and probable role of phytochrome in hair development, the density of hair on hypocotyls of WT and mutant tomato seedlings was observed by scanning electron microscopy.

Although morphologically WT, *au*, *au,hp* dark-grown seedlings appear similar, they possessed subtle morphological differences, such as density of hair on hypocotyl. Since the density of hair varies along the length of hypocotyl, the observations were uniformly restricted to the 1 cm region right below the hypocotyl hook.

Dark-grown *au* seedlings possessed little hair on hypocotyl (Fig. 4.6A), whereas *hp* (Fig. 4.5A) and WT (Fig. 4.4A) had a slightly more number of hair, indicating that *au* mutation caused a reduction in hypocotyl hair density. In seedlings transferred to RL for 24 h, of both WT (Fig. 4.4B) and *hp* (Fig. 4.5B), both density and length of hair increased considerably over the dark control. By contrast, *au* mutant (Fig. 4.6B) responded very sluggishly with initiation of very little hair and only a small change in hair length.

Wild-type

A. Dark



B. Red light

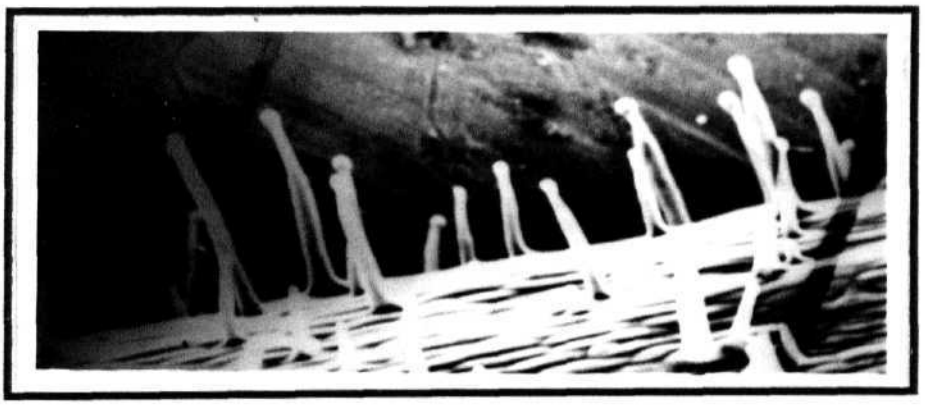
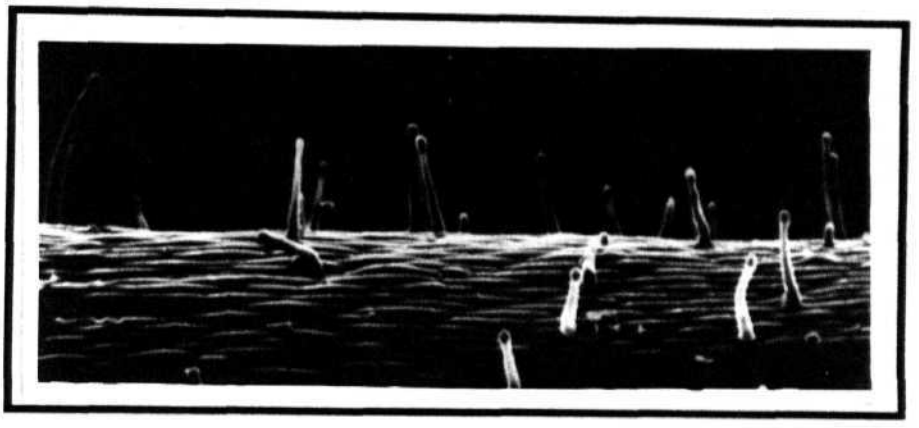


Figure 4.4: Scanning electron micrographs of hypocotyl hairs in dark (D) and red light (RL) grown wild-type tomato seedlings. 1 cm portion of hypocotyl below the hook region was excised from the seeseedlings grown in D for 120 h (A) and in 96 h D + 24 h RL (B) frozen in liquid nitrogen, and immediately observed (magnification 11 0X)

hp mutant

A. Dark



B. Red light

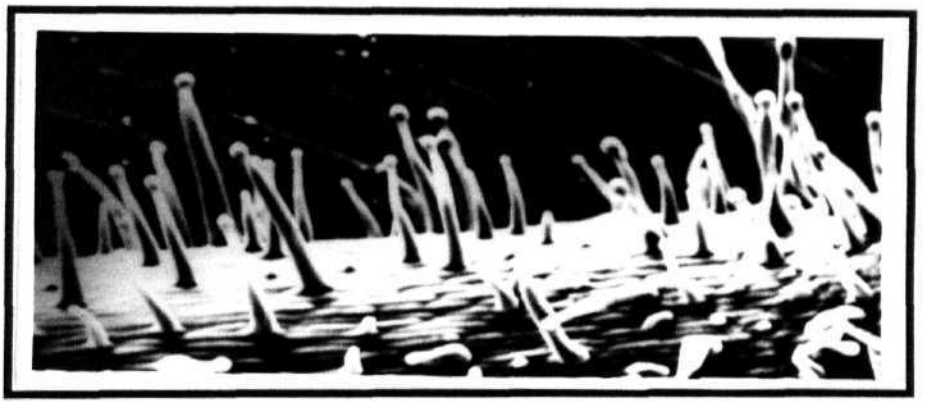


Figure 4.5: Scanning electron micrographs of hypocotyl hairs in dark (D) and red light (RL) grown *hp* tomato seedlings. 1 cm portion of hypocotyl below the hook region was excised from the seedlings grown in D for 120 h (A) and in 96 h D + 24 h RL (B) frozen in liquid nitrogen, and immediately observed (magnification 11 OX).

au mutant

A. Dark

B. Red light

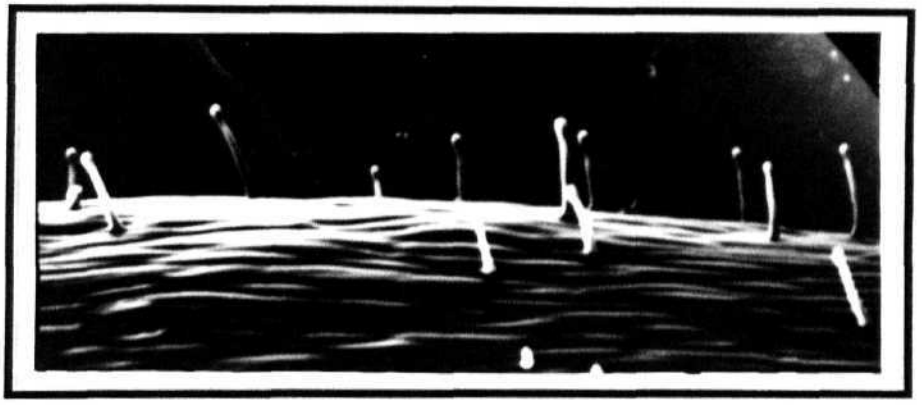


Figure 4.6: Scanning electron micrographs of hypocotyl hairs in dark (D) and red light (RL) grown *au* tomato seedlings. 1 cm portion of hypocotyl below the hook region was excised from the seedlings grown in D for 120 h (A) and in 96 h D + 24 h RL (B) frozen in liquid nitrogen, and immediately observed (magnification 110X)

4.2 Acquisition of photosynthesis

4.2.1 Accumulation of chlorophyll

The time course of total chlorophyll accumulation in cotyledons of mutant and WT seedlings is presented in Fig. 4.7. In seedlings transferred to RL, the chlorophyll accumulation is initiated at a steady rate in both *hp* and WT seedlings (Fig. 4.7A). By contrast, a perceptible increase in chlorophyll level in *au* seedlings can be seen only after 16 h of exposure to RL, and thereafter it sluggishly increases (Fig. 4.7A). By 48 h, *au* cotyledons have nearly five-fold less chlorophyll than WT and *hp*. In comparison, in *au* seedlings transferred to WL, chlorophyll accumulation proceeds normally (Fig. 4.7B), although *au* shows slower rate of accumulation than WT and *hp*. Unlike RL, there is no apparent lag in accumulation of chlorophyll in *au* seedlings grown under WL (Fig. **4.7B).**

4.2.2 Photosynthetic oxygen evolution

Development of photosynthesis in cotyledons after transfer of dark-grown seedlings to RL or WL was monitored by studying the time course of carbon dioxide dependent oxygen evolution using an oxygen monitor.

*4.2.2.1 **Photosynthetic** oxygen evolution under RL*

In seedlings grown under RL after transfer from dark, no oxygen evolution could be detected at 8 h of RL exposure in both mutants and WT. Thereafter, profiles of oxygen evolution followed different patterns. In WT oxygen evolution peaked at 24 h and remained at nearly the same level till 48 h (Fig. 4.8). In the *hp* mutant there was a increase of oxygen

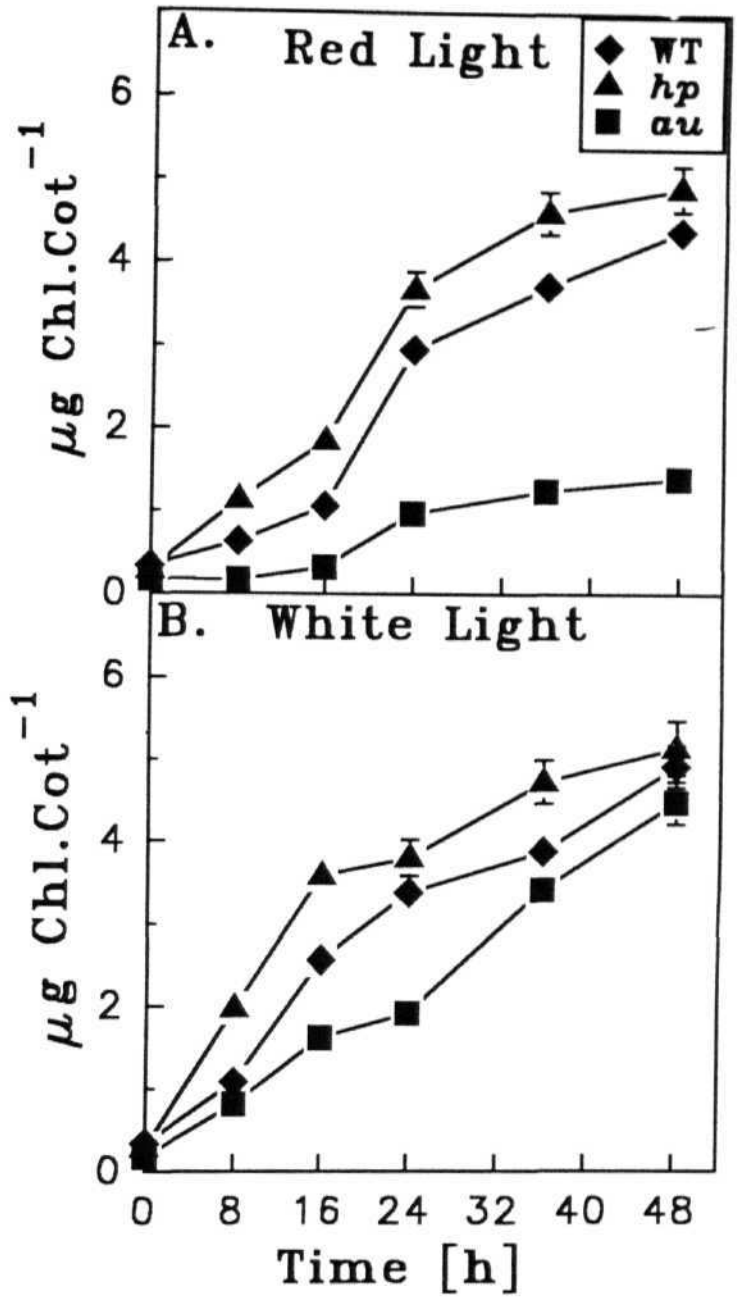


Figure 4.7: Time course of chlorophyll accumulation in cotyledons of tomato seedlings. Seedlings were grown in darkness upto 96 h from sowing and then transferred to continuous red light (A) and white light (B).

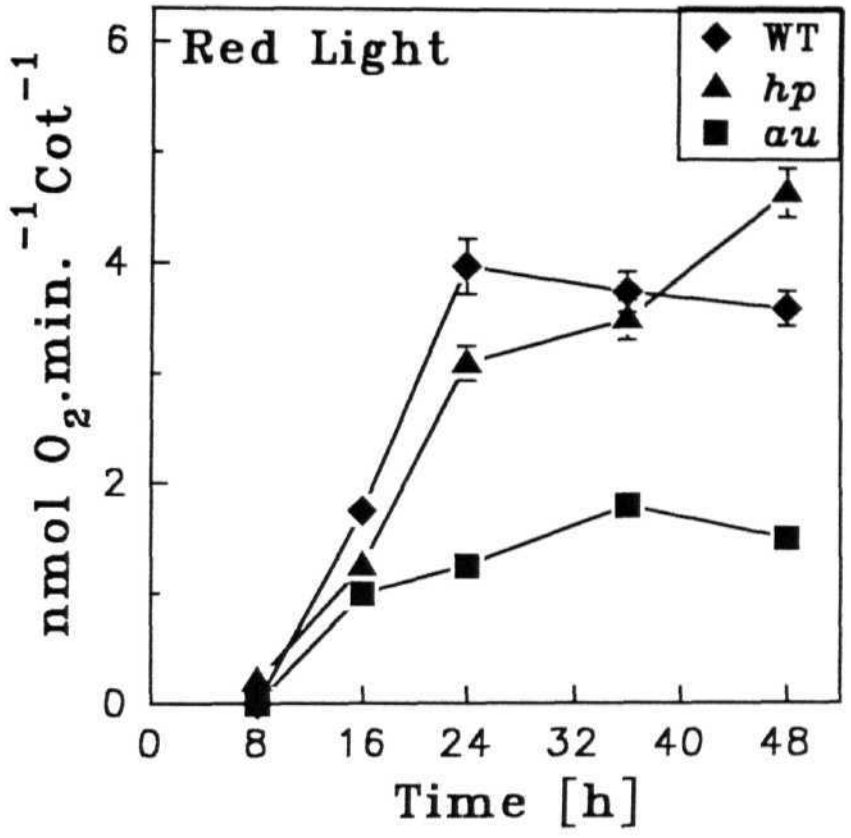


Figure 4.8 Time course of carbon dioxide dependent oxygen evolution in cotyledons of tomato seedlings. Seedlings were grown in darkness upto 96 h from sowing and then transferred to continuous red light. Cotyledons were excised at different time points and oxygen evolution was monitored *in vivo* using an oxygen monitor.

evolution till 48 h in RL. In contrast, the *au* mutant possessed a diminished evolution of oxygen under continuous RL

4.2.2.2 Photosynthetic oxygen evolution under WL

Unlike under RL exposure, in seedlings transferred to WL, there was considerable amount of oxygen evolution even at 8 h of WL exposure in both mutants and WT, but the magnitude of oxygen evolved was 3 to 4 fold higher in WT and *hp* when compared to *au* mutant. After 8 h, the profiles of oxygen evolution followed a similar pattern in both WT and *hp*, with a peak at 24 h and slightly decreased by 32 h thereafter it remained nearly constant till 48 h (Fig. 4.9). In contrast, in *au* mutant after 8 h there was a slow but steady increase in the evolution of oxygen upto 48 h when compared to WT and *hp* but it reached the WT levels by 48 h of WL exposure.

4.3 Photoinduction of phenylalanine ammonia lyase activity

Tomato mutant seedlings differ greatly with respect to photoinduction of anthocyanin and in potentiation by blue light (Adamse et al., 1989; Peters et al., 1991). In red, light-grown seedlings the maximum amount of anthocyanin is formed in *hp* seedlings and a little in WT, whereas the *au* mutant lacks accumulation of anthocyanin. Since PAL is one of the earliest enzymes in the anthocyanin biosynthetic pathway, we studied the effect of light on PAL levels of WT and mutant seedlings.

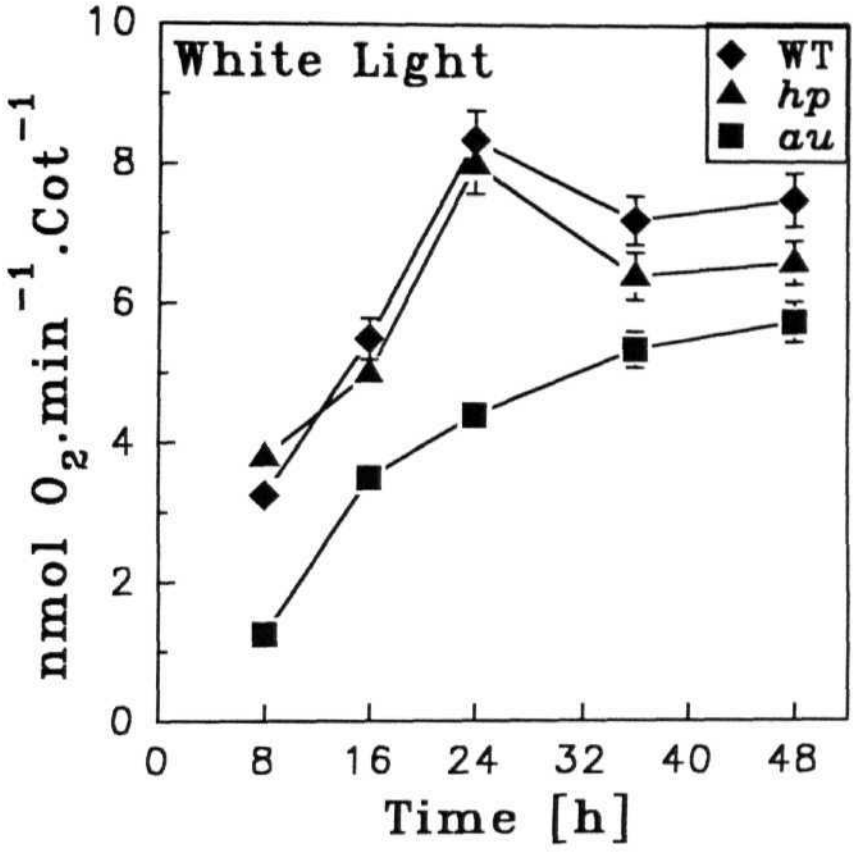


Figure 4.9 Time course of carbon dioxide dependent oxygen evolution in cotyledons of tomato seedlings. Seedlings were grown in darkness upto 96 h from sowing and then transferred to continuous white light. Cotyledons were excised at different time points and oxygen evolution was monitored *in vivo* using an oxygen monitor.

The influence of RL on the PAL activity in tomato seedlings was estimated by growing mutant *(au, hp,* and *au,hp)* and WT seedlings in continuous RL and in the darkness from the sowing. Fig.ure 4.10A shows that RL stimulated the PAL level in the cotyledons of the tomato mutants and WT seedlings. The magnitude of photoinduction was greatest in *hp,* decreased in WT and *au,hp,* and was least in the *au* seedlings. In contrast, no induction of PAL activity was detected in hypocotyls (Fig. 4.1 OB) of all the four genotypes. In seedlings transferred to 24 h continuous RL after 72 h in darkness, the level of PAL in the different genotypes followed a pattern similar to that of cotyledons of 96 h continuous RL grown seedlings (Fig. 4.11A) but here too no induction of PAL could be seen in the hypocotyl (Fig. 4.11B).

4.3.1 Time course of photoinduction of PAL activity

Figure 4.12 shows the time course of PAL activity in the tomato cotyledons in mutants and WT seedlings. Transfer from darkness to continuous RL resulted in a rapid enhancement in PAL activity, attaining a peak at 3 h after the onset of the RL. Thereafter, PAL activity gradually declined, nevertheless it was maintained at a significantly higher level than the dark levels after 24 h RL. In the case of *hp* the PAL activity after attaining a peak level declined only slightly compared to other genotypes. Since, in all cases, within 1 h of exposure to RL a significant induction of PAL activity is noticeable, it is apparent that photoinduction of PAL is preceded by only a very short lag phase.

The time course of photoinduction of PAL level in the hypocotyl followed a different pattern than that in the cotyledons (Fig. 4.13). In the

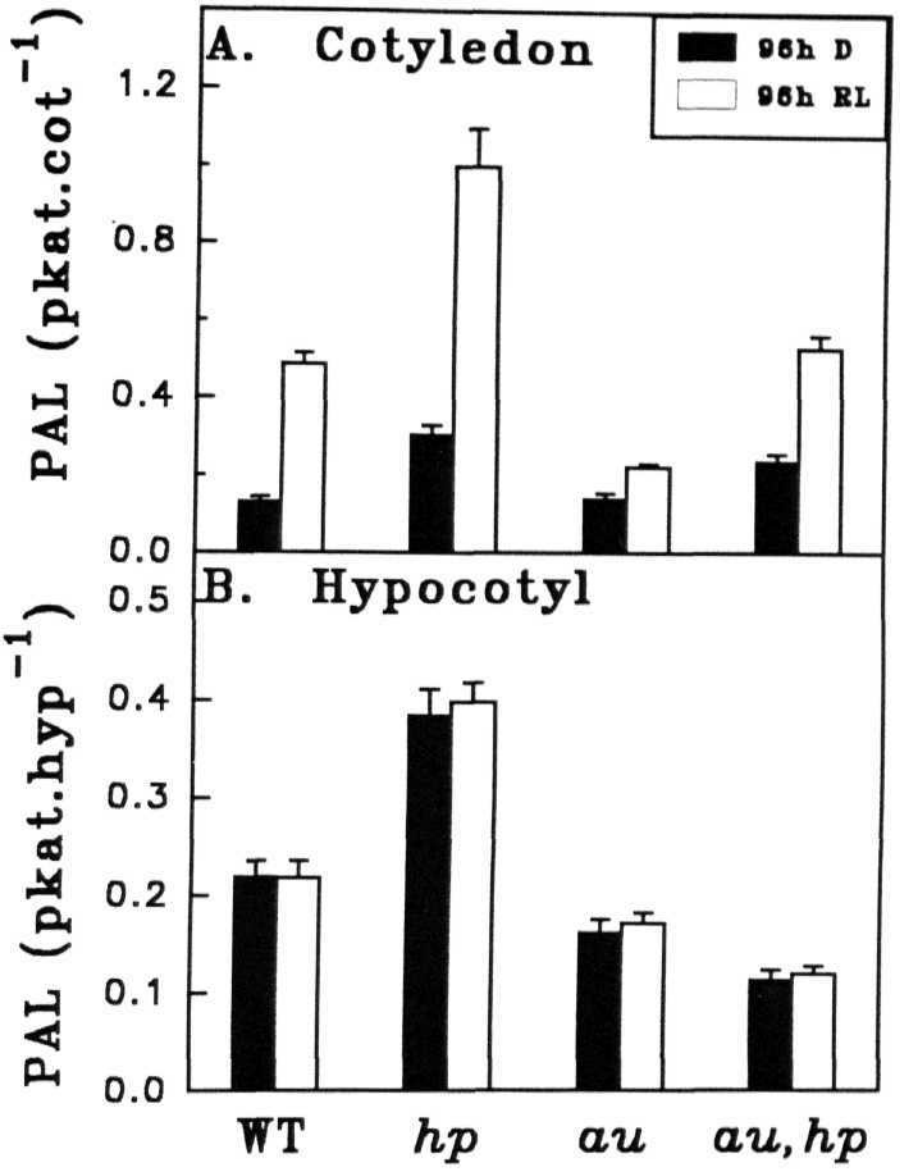


Figure 4.10 PAL activity in cotyledons (A) and hypocotyls (B) of tomato seedlings. The PAL activity was measured at 96 h from sowing in wild-type and mutant seedlings grown in complete darkness (D) and continuous red light (RL).

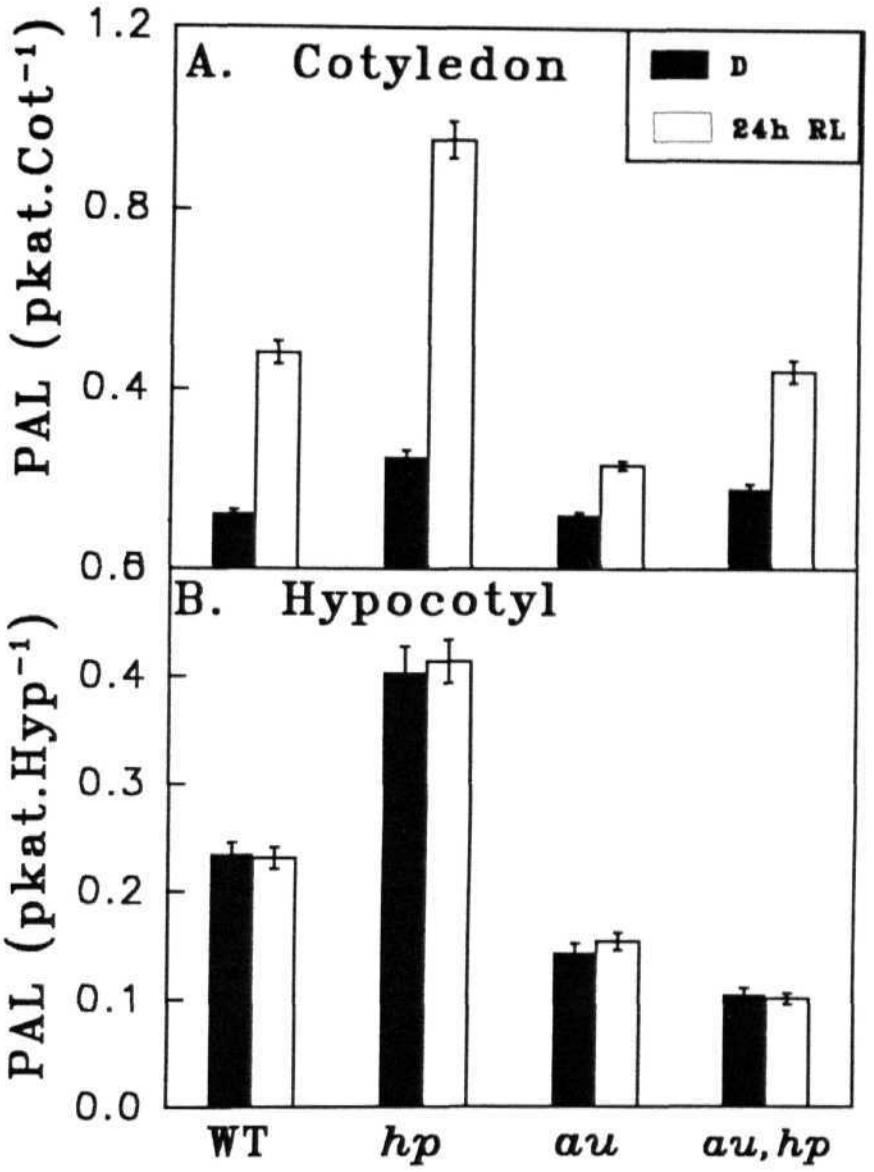


Figure 4.11 PAL activity in cotyledons (A) and hypocotyls (B) of tomato seedlings. The PAL activity was measured at 96 h from sowing in wild-type and mutant seedlings grown in complete darkness (D), and 72 h D + 24 h RL.

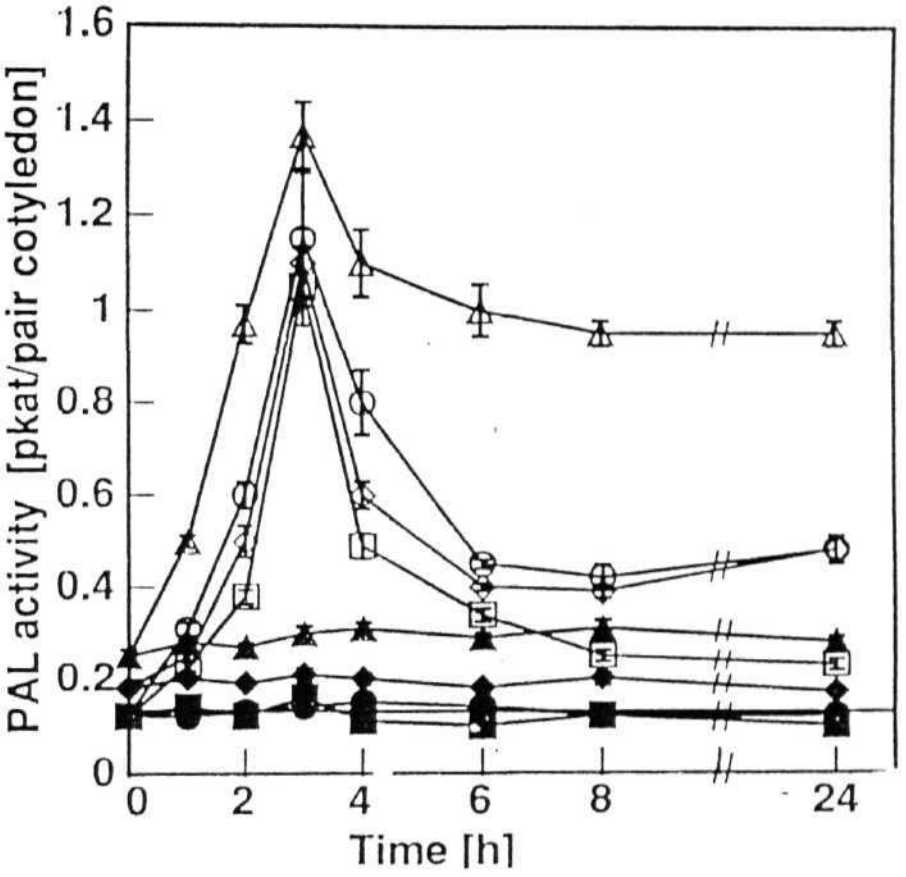


Figure 4.12 Time course of PAL activity in cotyledons of tomato seedlings. Seedlings were grown in darkness upto 96 h from sowing and then were transferred to continuous RL (open symbols). The control seedlings were maintained in continuous darkness (closed symbols).

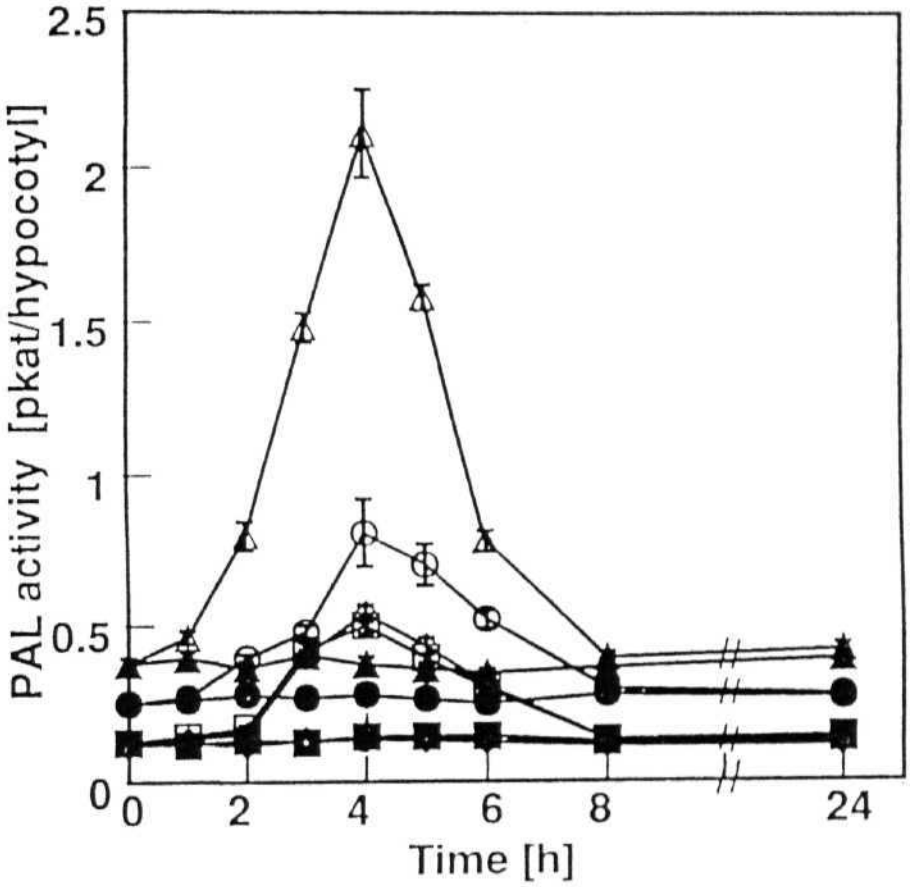


Figure 4.13 Time course of PAL activity in hypocotyls of tomato seedlings. Seedlings were grown in darkness upto 96 h from sowing and then transferred to continuous RL (open symbols). The control seedlings were maintained in continuous darkness (closed symbols).

hp mutant, PAL levels increased at a more rapid rate than in the other mutants and WT seedlings, and were five times higher 4 h after the onset of RL irradiation. Thereafter, the level of PAL gradually declined and by 8h, after the onset of RL, PAL activity reached the dark control levels for all the genotypes. Hence we could not detect PAL activity in the hypocotyl after more than 8 h of continuous irradiation.

In comparison to the *hp* mutant, the magnitude of PAL induction in WT, *au* and *au,hp* seedlings was much less, but even in these seedlings the photoinduction of PAL lasted only for a period of 8 h with a peak level at 4 h after the onset of RL. In comparison to cotyledons, photoinduction of PAL activity in hypocotyl shows a perceptible lag period of about 1 h and increase in PAL activity becomes evident only after that period.

4.3.2 Phytochrome-mediated PAL photoinduction

The participation of phytochrome in the photoinduction of PAL was investigated by the R/FR reversibility experiments. Figure 4.14 and 4.15 show that in tomato seedlings RL acting via phytochrome significantly increase PAL activity in both cotyledons and hypocotyl. A single pulse of RL is nearly as effective as continuous RL, and if it is followed by a FR pulse, the effect of the RL pulse on PAL induction is significantly negated. FRL alone also induced about 1.5 to 2 fold increase in the PAL level in the cotyledons and in the hypocotyl. In the cotyledons, there was little difference in the magnitude RL-mediated induction of PAL level between mutants and WT seedlings. In contrast, in the hypocotyl of the *hp* mutant (Fig. 4.15) the RL-mediated induction

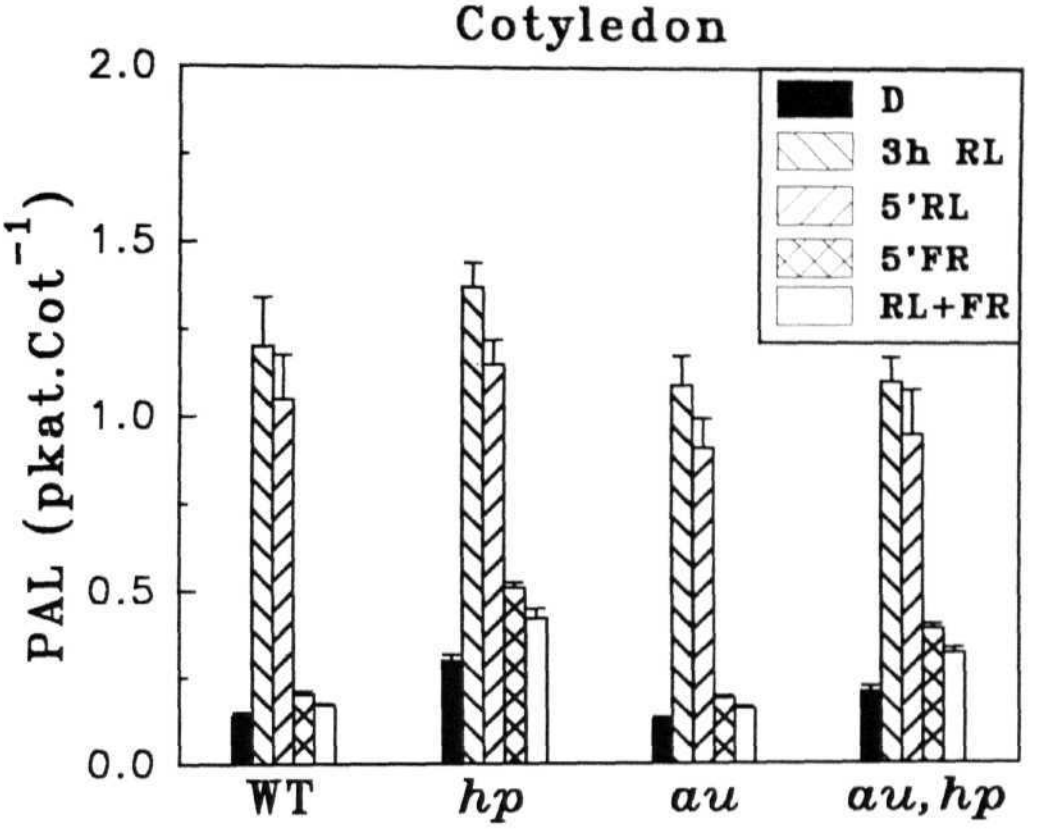


Figure 4.14 Effect of 5 min red light (RL) and far-red light (FRL) irradiation on PAL activity in cotyledons of tomato seedlings. Seedlings were grown in darkness (D) for 96 h from sowing and then subjected to 3 h RL. A set of seedlings were exposed to RL (5'), FRL (5'), and RL followed by FRL irradiation, and then were transferred back to dark for the next 3 h. The control seedlings remained in D. The enzyme activity was assayed at 99 h from sowing.

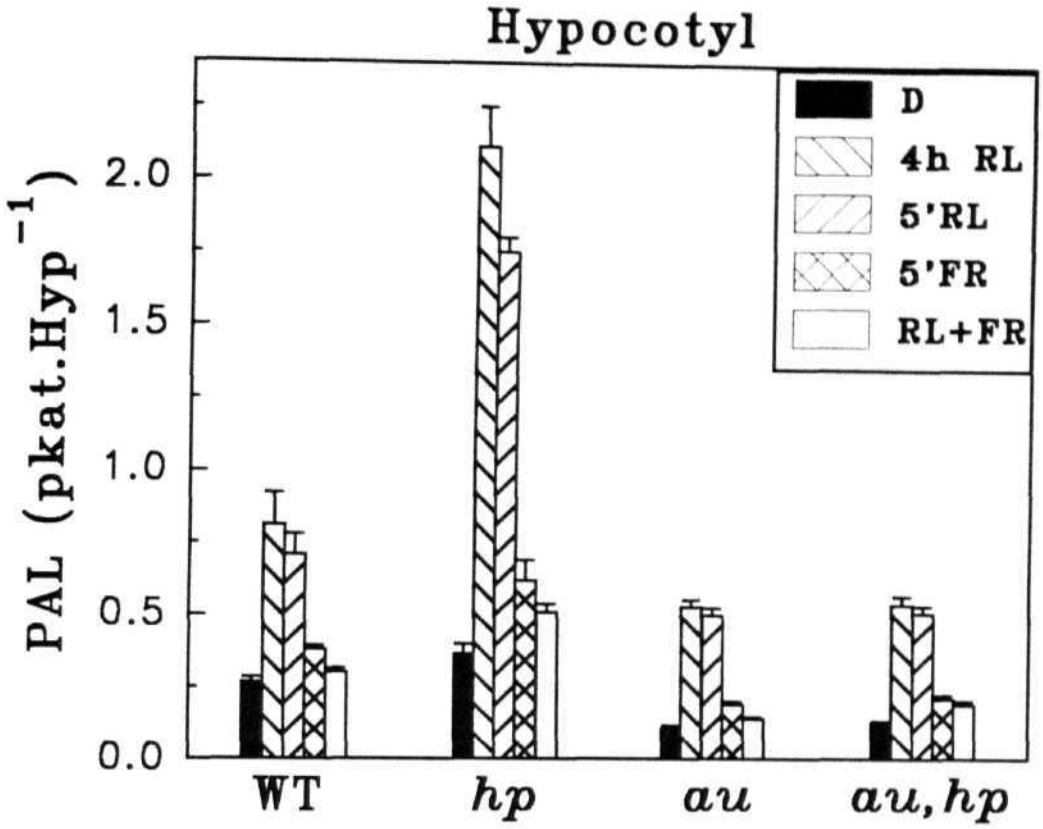


Figure 4.15 Effect of 5 min red light (RL) and far-red light (FRL) irradiation on PAL activity in hypocotyls of tomato seedlings. Seedlings were grown in darkness (D) for 96 h from sowing and then subjected to 4 h RL. Seedlings were exposed to RL (5'); FRL (5'); and RL followed by FRL irradiation and were then transferred back to dark for the next 4 h. The control seedlings remained in D. The enzyme activity was assayed at 100 h from sowing.

of **PAL** level was much greater in magnitude than that of the WT. However, RL-mediated induction is lower in magnitude in the *au* and the *au,hp* mutant hypocotyls. Interestingly, in the *hp* mutant, both in the hypocotyl and cotyledons the level of PAL in seedlings **grown in** absolute darkness was 1.5 to 2 fold higher than in the corresponding WT seedlings, while in dark-grown *au* seedlings the **PAL** level was equalent or lower than WT in the cotyledons and in the hypocotyl respectively.

4.3.3 Photoinduction of PAL is dependent on RNA and protein synthesis

The rapid photoinduction of PAL in cotyledons and hypocotyl can arise either as a consequence of modulation of its activity or by an increase in the rate of its *de nova* synthesis, or by reduction in turnover of PAL protein. To test this, the dependence of photoinduction of PAL on RNA and protein synthesis in seedlings of all tomato genotypes was investigated by using inhibitors of these processes, i.e., actinomycin-D and cordycepin for RNA synthesis inhibition and cycloheximide and puromycin for protein synthesis inhibition.

The seedlings were treated with above inhibitors two hours before transferring to RL. The application of these inhibitors prior to the onset of RL irradiation significantly inhibited the photoinduction of PAL in both cotyledons and hypocotyls of WT (Fig. 4.16), *hp* (Fig. 4.17), *au* (Fig. 4.18) and *au,hp* (Fig. 4.19) mutant seedlings. The extent of inhibition is nearly three to four fold in both cotyledons and hypocotyls with **all** the four inhibitors used.

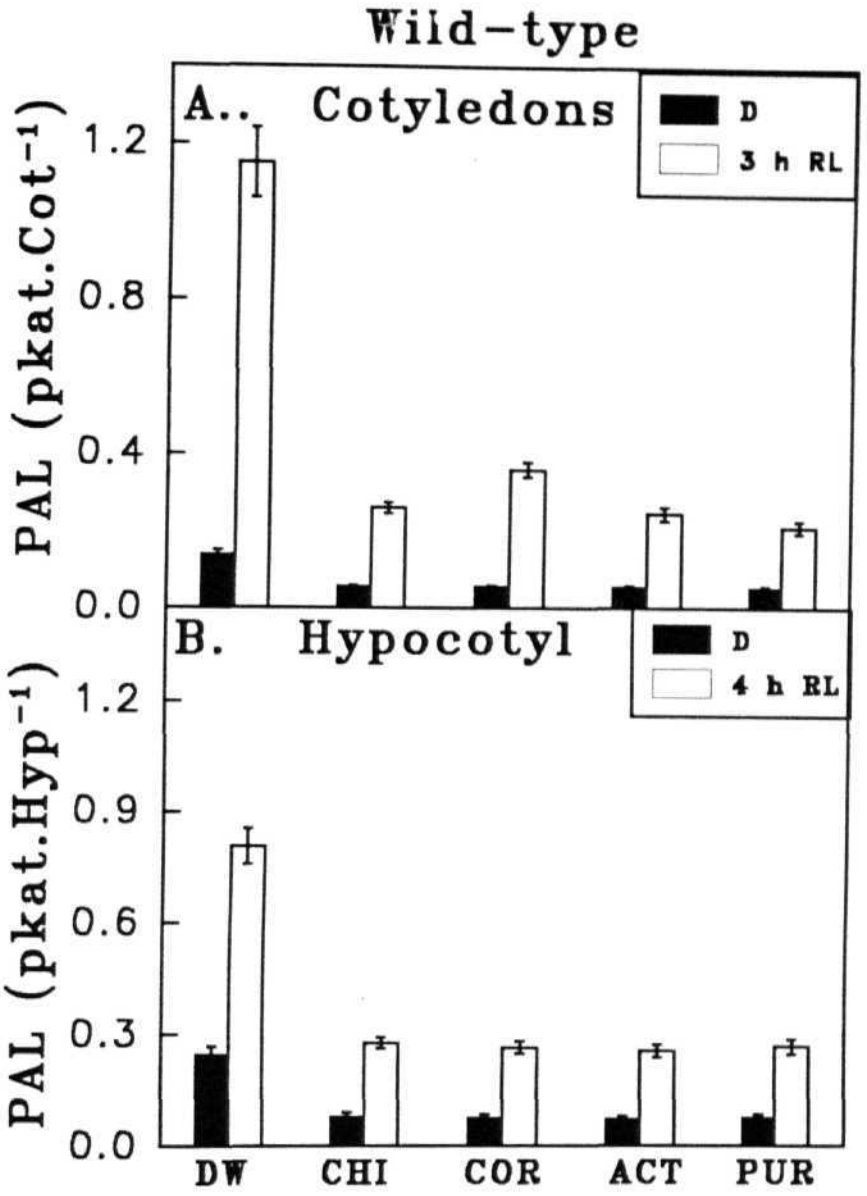


Figure 4.16 Effect of inhibitors of RNA [ACT (Actinomycin-D-100 µg ml-1); COR (cordycepin-200 µg ml^{-1})] and protein [CHI (cycloheximide-25 µg ml^{-1}); PUR (puromycin-200 µg ml^{-1})] synthesis on photoinduction of PAL activity in cotyledons (A) and hypocotyls (B) of wild type. Seedlings grown in darkness (D) for 96 h from sowing were transferred to continuous red light (RL) and PAL activity was measured after 3 h and 4 h of continuous RL in cotyledons and hypocotyls respectively. The seedlings were treated with the above inhibitors 2 h before transferring to RL. The control seedlings in D were similarly treated with distilled water or inhibitors.

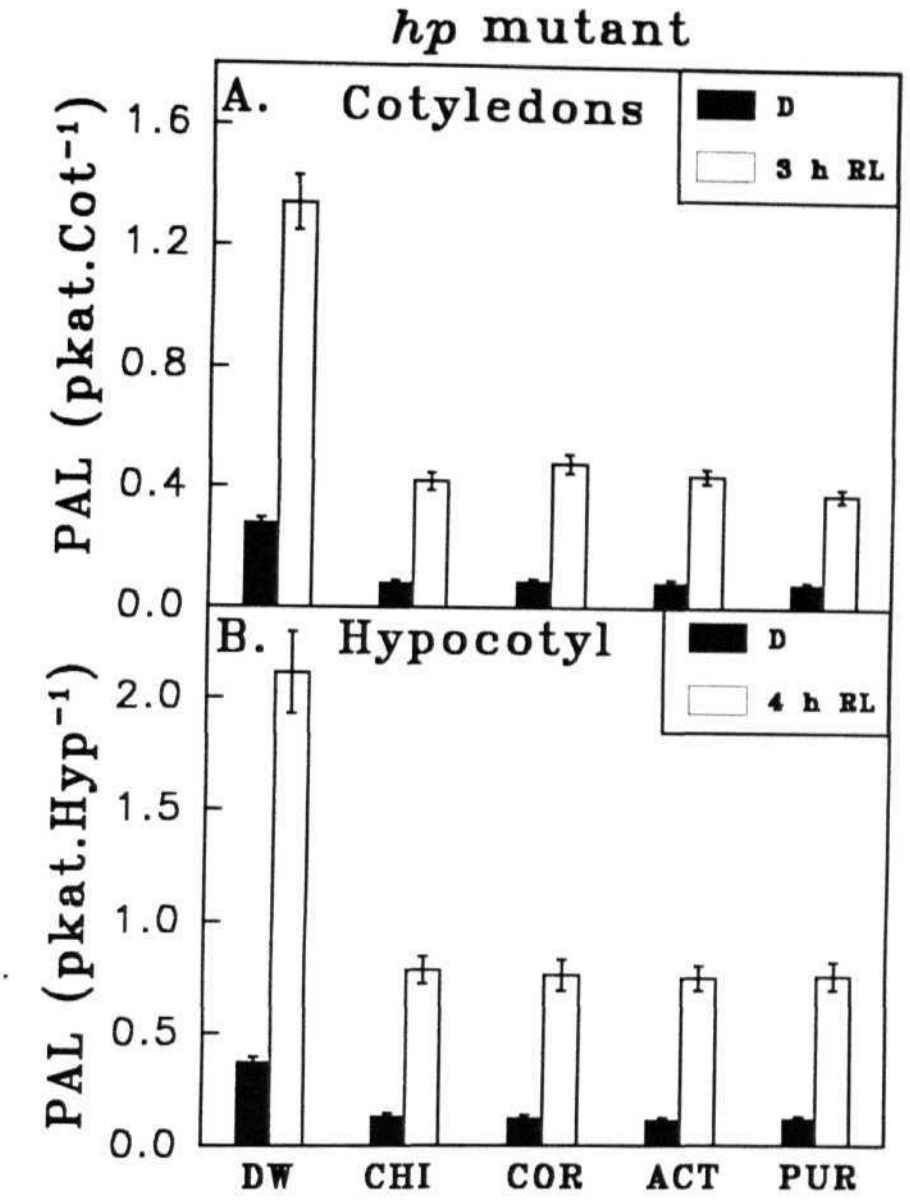


Figure 4.17 Effect of inhibitors of RNA [ACT (Actinomycin-D-100 µg ml^{-1}); COR (cordycepin-200 µg ml^{-1})] and protein [CHI (cycloheximide-25 µg ml^{-1}); PUR (puromycin-200 µg ml^{-1})] synthesis on photoinduction of PAL activity in cotyledons (A) and hypocotyls (B) of *hp* mutant. Seedlings grown in darkness (D) for 96 h from sowing were transferred to continuous red light (RL) and PAL activity was measured after 3 h and 4 h of continuous RL in cotyledons and hypocotyls respectively. The seedlings were treated with the above inhibitors 2 h before transferring to RL. The control seedlings in D were similarly treated with distilled water or inhibitors.

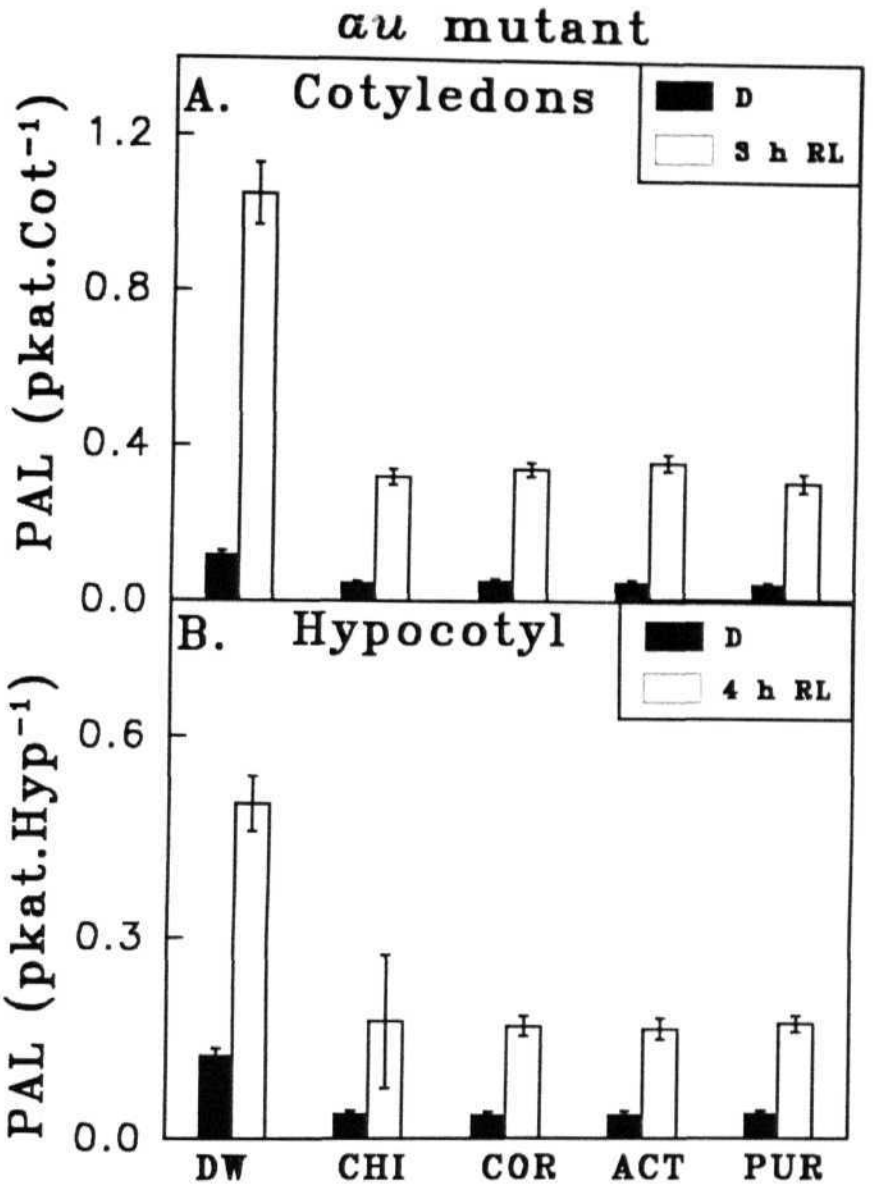


Figure 4.18 Effect of inhibitors of RNA [ACT (Actinomycin-D-100 µg ml^{-1}); COR (cordycepin-200 µg ml^{-1})] and protein [CHI (cycloheximide-25 µg ml^{-1}); PUR (puromycin-200 µg ml^{-1})] synthesis on photoinduction of PAL activity in cotyledons (A) and hypocotyls (B) of *au* mutant. Seedlings grown in darkness (D) for 96 h from sowing were transferred to continuous red light (RL) and PAL activity was measured after 3 h and 4 h of continuous RL in cotyledons and hypocotyls respectively. The seedlings were treated with the above inhibitors 2 h before transferring to RL. The control seedlings in D were similarly treated with distilled water or inhibitors.

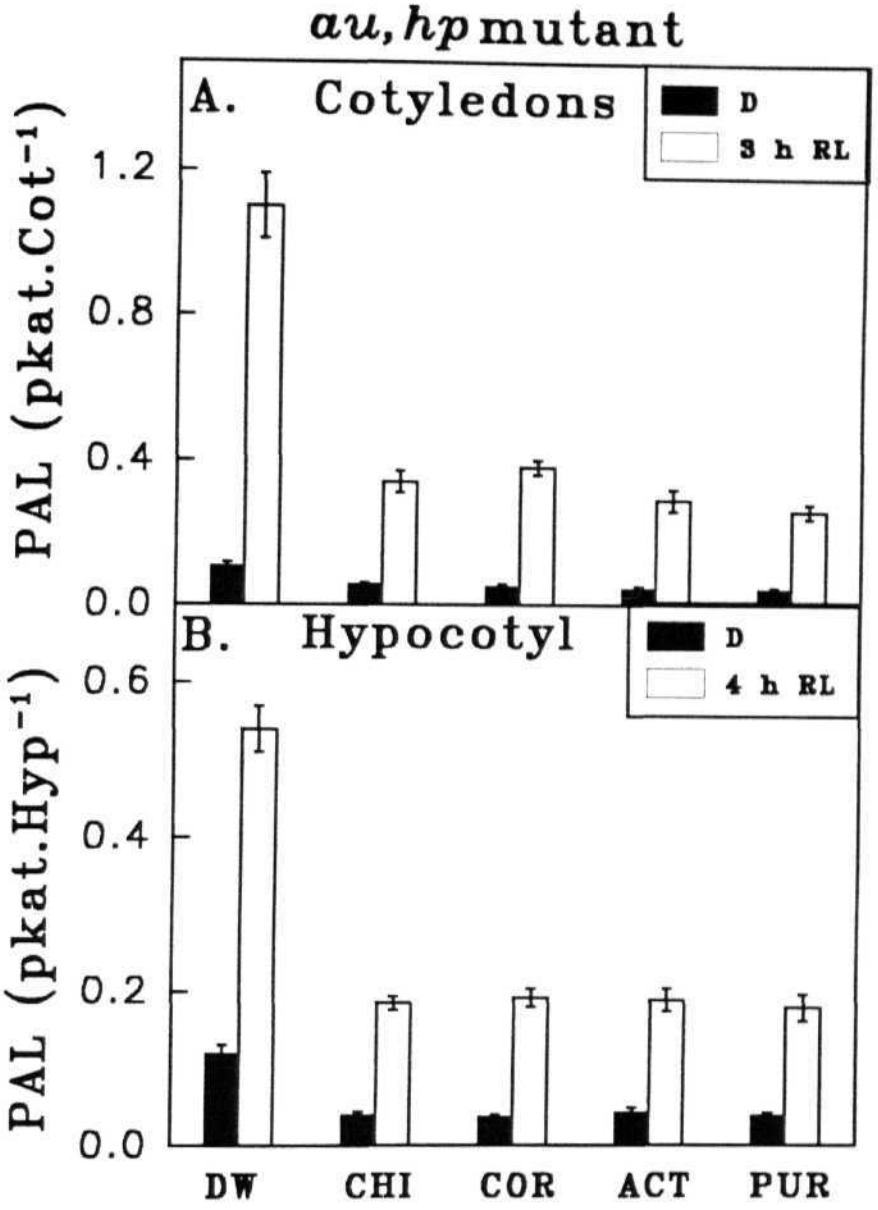


Figure 4.19 Effect of inhibitors of RNA [ACT (Actinomycin-D-100 µg ml-1); COR (cordycepin-200 µg ml^{-1})] and protein [CHI (cycloheximide-25 µg ml^{-1}); PUR (puromycin-200 µg ml^{-1})] synthesis on photoinduction of PAL activity in cotyledons (A) and hypocotyls (B) of *au,hp* mutant. Seedlings grown in darkness (D) for 96 h from sowing were transferred to continuous red light (RL) and PAL activity was measured after 3 h and 4 h of continuous RL in cotyledons and hypocotyls respectively. The seedlings were treated with the above inhibitors 2 h before transferring to RL. The control seedlings in D were similarly treated with distilled water or inhibitors.

4.3.4 PAL photoinduction results from *de novo* synthesis of PAL proteins

Earlier studies, using specific inhibitors of protein and RNA synthesis, indicated that PAL photoinduction is directly a result of an increase in the rate of its synthesis leading to an increase in the level of the PAL protein. The *in vivo* level of PAL protein was therefore monitored using polyclonal antibodies raised against tobacco PAL. Since both tobacco and tomato belong to same family of Solanacea the PAL antibodies are expected to recognize the tomato PAL protein too.

The time course of PAL induction using tobacco PAL antibody revealed that photostimulated increase in level of PAL protein can be detected within 1 h of RL exposure, and is perceptible till 6 h. Thereafter, PAL levels decrease to levels equal to dark controls by 12 h in both cotyledons and hypocotyl of *au* (Fig. 4.21) and WT (Fig. 4.20) seedlings. In contrast, in *hp* seedlings also accumulation of protein starts within 1 h of RL exposure but unlike *au* and WT it continues till 12 h of RL irradiation (Fig. 4.22) in both cotyledons and hypocotyls. Thereafter, it decreases by 24 h but, the amount of protein accumulated is considerably higher when compared to respective dark control, in both the organs of *hp* mutant. The accumulation of more amounts of PAL protein in *hp* is consistent with the high amounts of anthocyanin that accumulate in this mutant (Table 4.1). The above observations confirm that the RL-mediated induction of PAL activity in tomato seedlings is due to accumulation of PAL protein as a result of *de novo* synthesis.

Wild type

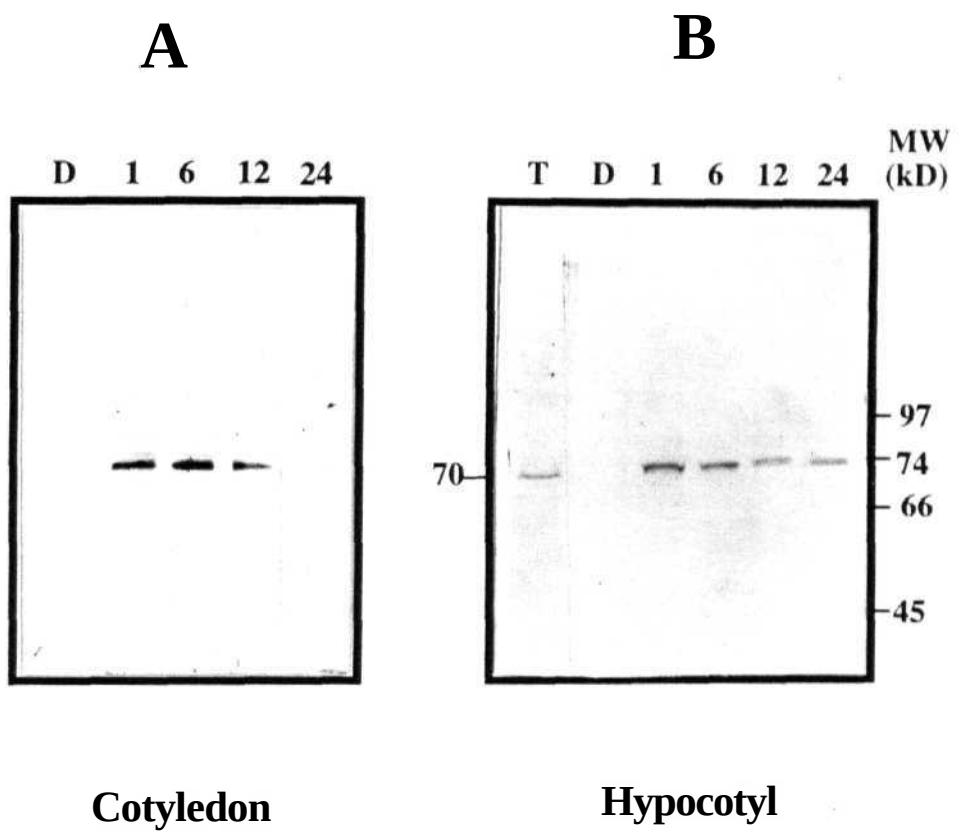


Figure 4.20: Time course of PAL level in cotyledons (A) and hypocotyl (B) of wild type tomato, after transfer to continuous RL. Seedlings were grown in darkness upto 96 h from sowing and then were transferred to RL. At the time point indicated above, seedlings were harvested, and cotyledons and hypocotyls were excised. The amount of PAL protein was detected after SDS-PAGE and electroblotting on PVDF paper. The blot was probed by using a primary antibody raised against tobacco PAL. Equal amounts of protein was loaded in all gel tracks to facilitate the comparison of relative levels of PAL protein. The track labelled T showed the PAL extracted from tobacco, which was included for comparison.

au mutant

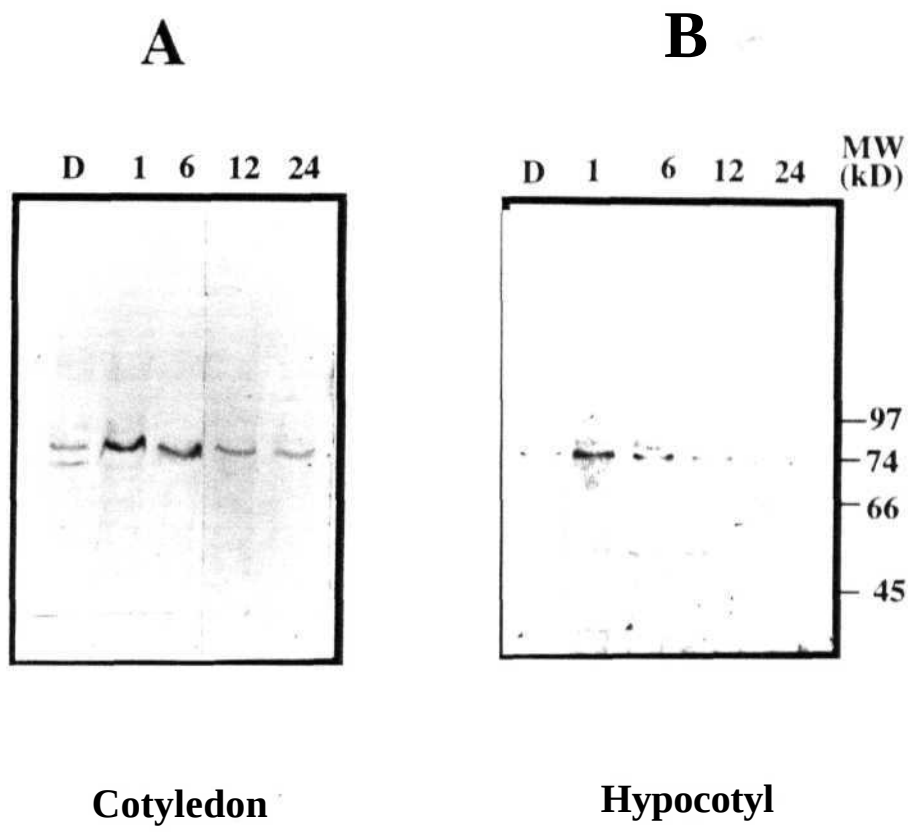


Figure 4.21: Time course of PAL level in cotyledons (A) and hypocotyl (B) of *au* mutant of tomato, after transfer to continuous RL. Seedlings were grown in darkness upto 96 h from sowing and then were transferred to RL. At the time point indicated above, seedlings were harvested, and cotyledons and hypocotyls were excised. The amount of PAL protein was detected after SDS-PAGE and electroblotting on PVDF paper. The blot was probed by using a primary antibody raised against tobacco PAL. Equal amounts of protein was loaded in all gel tracks to facilitate the comparison of relative levels of PAL protein.

hp mutant

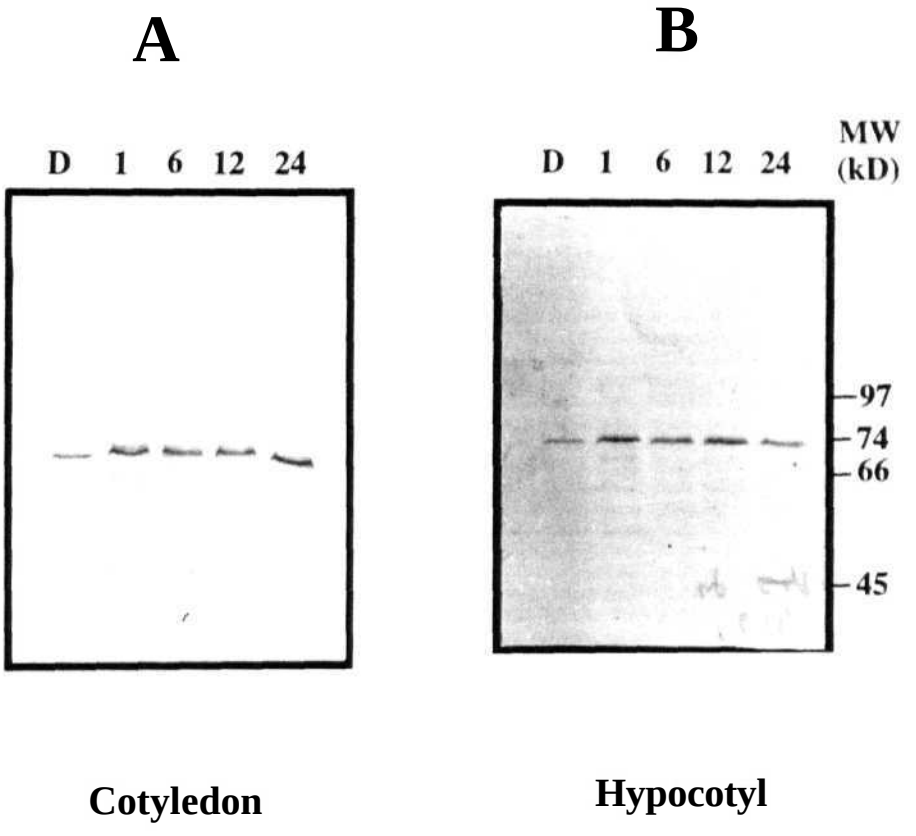


Figure 4.22: Time course of PAL level in cotyledons (A) and hypocotyl (B) of *hp* mutant of tomato, after transfer to continuous RL. Seedlings were grown in darkness upto 96 h from sowing and then were transferred to RL. At the time point indicated above, seedlings were harvested, and cotyledons and hypocotyls were excised. The amount of **PAL** protein was detected after SDS-PAGE and electroblotting on PVDF paper. The blot was probed by using a primary antibody raised against tobacco **PAL.** Equal amounts of protein was loaded in all gel tracks to facilitate the comparison of relative levels of **PAL** protein.

Table 4.1: *The anthocyanin content of tomato seedlings grown in the presence (+) or absence (-) of α-aminoxy, fi-phenylpropionic acid*

Anthocyanin content (A_{535} nm)/10 organs								
	Cotyledons				Hypocotyl			
	96 **hD**		72 h D + 24 h RL		96 **hD**		72 h D +24 h RL	
	-	+	-	+	-	+	-	+
WT	2	1	8	2	3	1	46	16
hp	3	1	69	12	4	1	555	155
au	0	0	0	0	0	0	2	1
au,hp	0	0	0	0	0	0	3	1

Seedlings were grown in the darkness (D) at 72 h from sowing, a set of seedlings were transferred to continuous RL. The anthocyanin content was measured in hypocotyl and cotyledons separately after 24 h continuous RL. The seedlings were treated with a-aminoxy, β-phenyl propionic acid 2 h before transffering to RL. The control seedlings were similarly treated with distilled water. The standard error for the anthocyanin values presented in the table was <5%.

4.4 Anthocyanin biosynthesis is independent of photoinduction of PAL

PAL is notionally the first enzyme in the biosynthetic pathway which leads to biosynthesis of anthocyanin and other flavonoids. It is assumed that photoinduction of biosynthesis of anthocyanin is preceded by an induction in the level of PAL enzyme.

The possible role of the PAL in mediating anthocyanin biosynthesis in WT and mutants was investigated by using a PAL specific inhibitor α-aminoxy, β-phenylpropionic acid (Amrhein and Godeke 1977). This inhibitor is a substrate analog for phenylalanine and inhibits the activity of PAL by binding to its active site. The seedlings were treated with the above inhibitors two hours before transferring to RL. Figure 4.23 shows that treatment with this inhibitor brings about a reduction of the PAL activity even in darkness and completely abolishes the photoinduced increase in PAL activity in WT and mutant seedlings.

Whether the inhibitor induced PAL inhibition also leads to an inhibition of anthocyanin level was examined by estimating anthocyanin content of tomato seedlings transferred to RL for 24 h after growing for 72 h in dark. It was observed that there is very little anthocyanin accumulation in cotyledons of WT and hp seedlings compared to hypocotyls and that hp seedlings produce about eight to ten fold more than WT seedlings. In contrast, there is obsolutely no anthocyanin formation in au and au,hp seedlings (Table 4.1). The estimation of anthocyanin level in cotyledons and the hypocotyl of tomato seedlings treated with α-aminoxy, β-phenylpropionic acid revealed that the

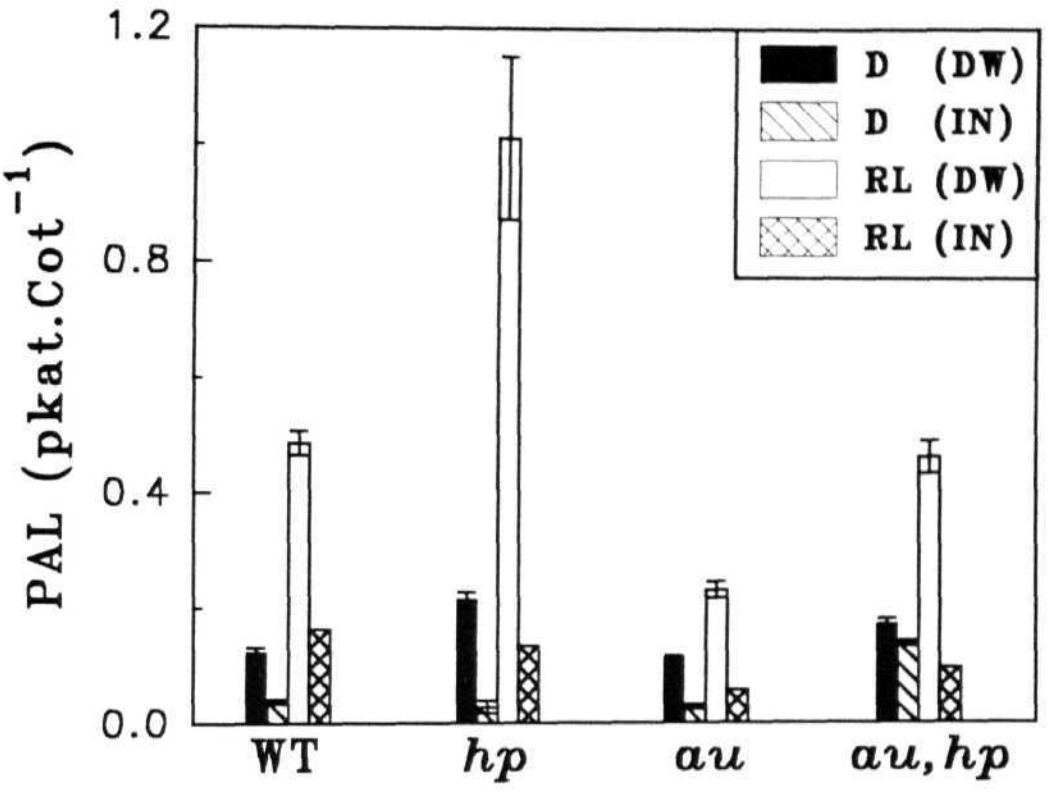


Figure 4.23: Effect of α-aminoxy,β-phenylpropionic acid on photoinduction of PAL activity. Tomato seedlings were grown in darkness (D). At 72 h from sowing, a set of seedlings was transferred to continuous red light (RL). PAL activity was measured in the cotyledons after 24 h of continuous RL. The seedlings were treated with the above inhibitor (0.4 mM) (IN) 2 h before transferring to RL. The control seedlings were similarly treated with distilled water (DW).

inhibitor treatment to a large extent also abolished RL-mediated induction of anthocyanin in hp and WT seedlings (Table 4.1).

4.5 Photoinduction of other cytosolic enzymes and a plastidic enzyme

Earlier experiments indicated that inspite of being deficient of spectrally active phytochrome, *au* seedlings retained a significant RL-mediated photoinduction of a cytosolic enzyme PAL. However, at the same time it was reported that under RL these seedlings fails to induce transcripts for nuclear-encoded plastidic enzymes under similar conditions. In view of this, we examined the possibility if observed photoinduction of enzyme in *au* seedlings is restricted only to cytosolic enzymes and not to plastidic enzymes. For comparative purpose we examined photoinduction of two cytosolic enzymes NR and amylase and photoinduction of a plastidic enzyme such as NiR in cotyledons and hypocotyls of WT and mutant seedlings.

4.5.1 Photoinduction of cytosolic Nitrate reductase and amylase activities

The involvement of phytochrome in the photoinduction of NR (Fig. 4.25) and amylase (Fig. 4.24) activities in tomato seedlings was checked by examining effect of brief RL and FRL pulses on enzyme activity in cotyledon and hypocotyl. Figures 4.24 and 4.25 show that a brief pulse of RL significantly increased NR and amylase activities in both cotyledons and hypocotyls of WT and *hp*. The effect of the RL pulse was significantly negated when it was followed by a FR pulse. However, In comparison with continuous RL irradiation the effect of a

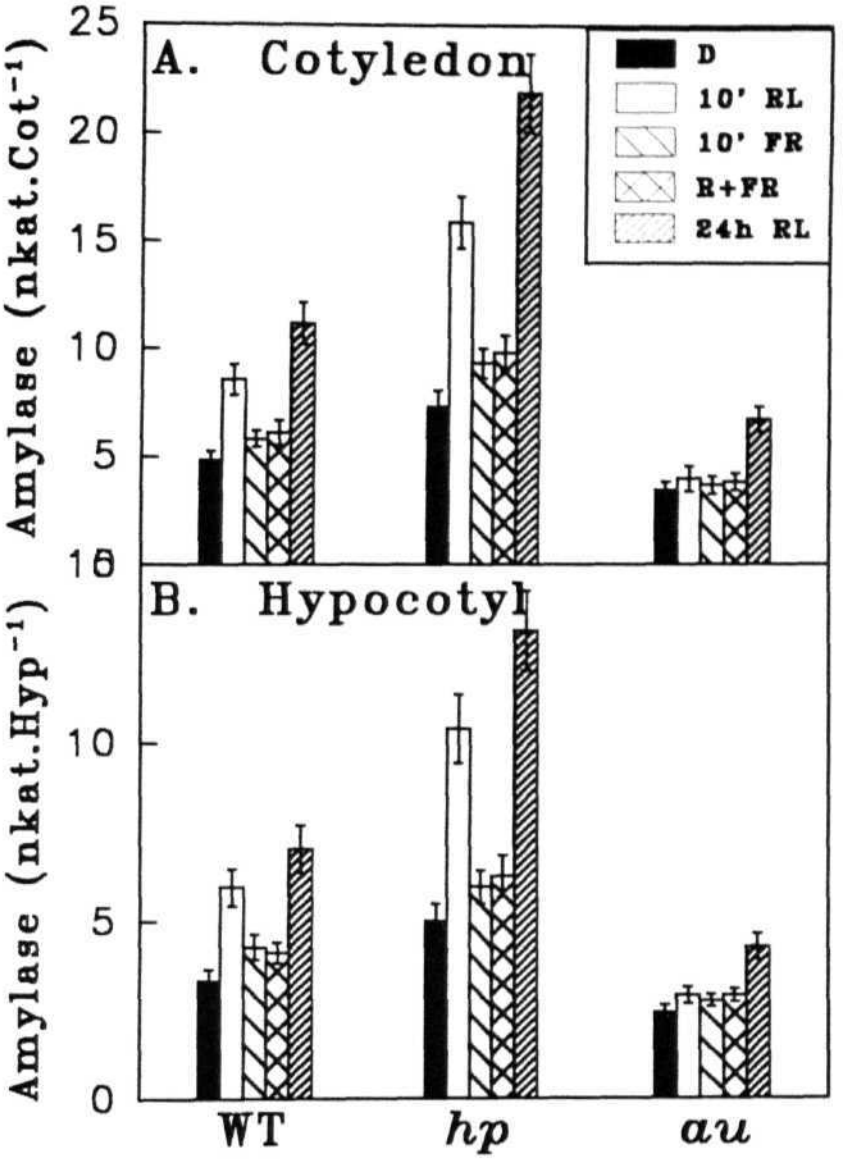


Figure 4.24: Effect of 10 min red light (RL) and far-red (FRL) irradiation on amylase activity in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown in darkness for 96 h from sowing, and then were subjected to continuous RL, brief RL or FRL irradiation as described in the figure. The control seedlings remained in darkness (D). Enzyme activity was estimated at 120 h from sowing.

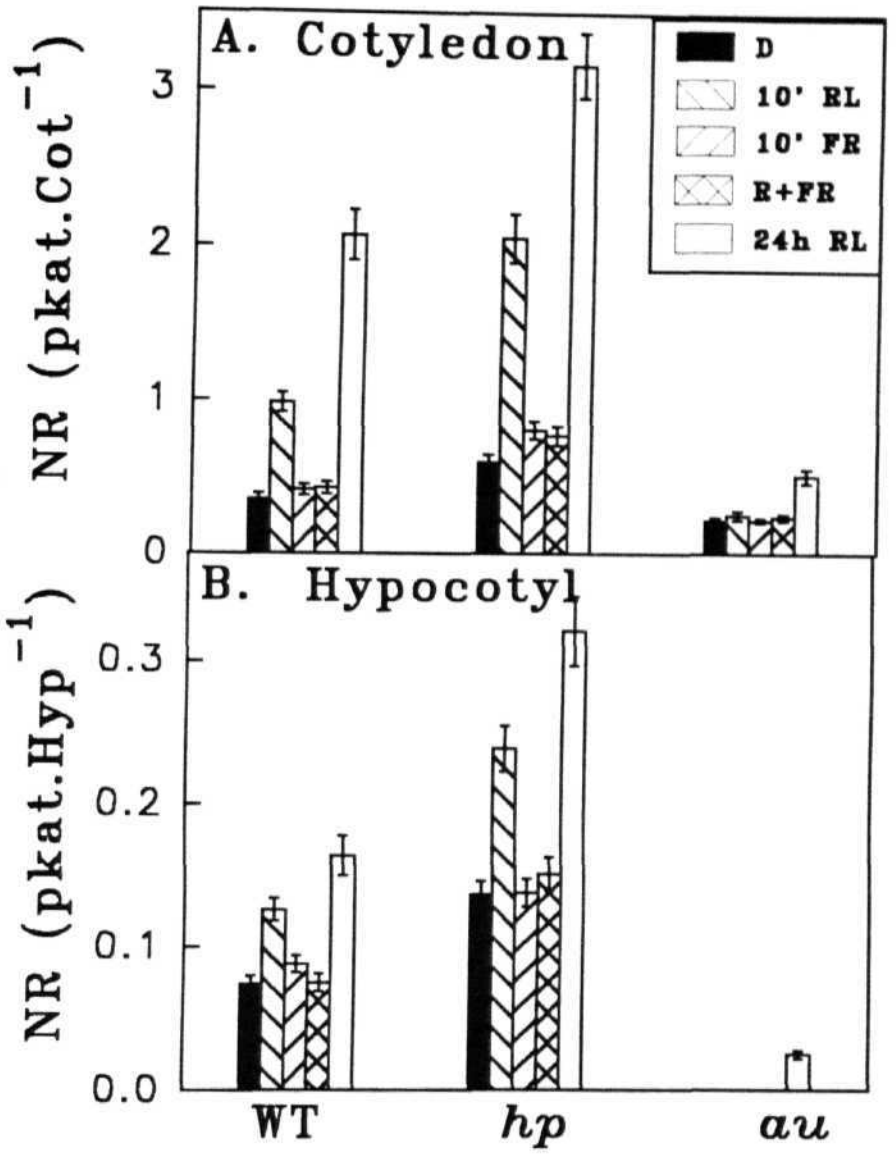


Figure 4.25: Effect of 10 min red light (RL) and far-red (FRL) irradiation on nitrate reductase activity in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown in darkness for 96 h from sowing, and then subjected to continuous RL, brief RL or FRL irradiation as described in the figure. The control seedlings remained in darkness (D). Enzyme activity was estimated at 120 h from sowing.

brief RL pulse on photoinduction of enzymes was weak. In general, the magnitude of photoinduction of enzymes was higher in *hp* than in WT. By contrast in *au* mutant brief RL pulse failed to induce activity of NR and amylase. At the same time exposure of *au* seedlings to continuous RL photoinduced activity of both NR and amylase (Figs. 4.24 and 4.25). In case of *au* hypocotyl the NR activity was close to the level of detection limit in dark-grown seedlings. However, it could be induced by continuous RL.

4.5.2 Photoinduction of plastidic nitrite reductase activity

The role of phytochrome in the photoinduction of NiR activity was also investigated by R/FR reversible experiments. A brief pulse of RL has also significantly increased NiR activity in both cotyledons and hypocotyls of WT and *hp* and the effect of it was negated when followed by a FR pulse (Fig. 4.26). The effect of RL pulse was weak when compared to continuous RL irradiation. By contrast, in the case of *au* mutant a brief RL pulse and continuous RL irradiation failed to induce NiR activity in both cotyledons (Fig. 4.26A) and hypocotyl (Fig..4.26B) though continuous RL induced NR (Fig. 4.25) and amylase (Fig. 4.24) activity in it.

4.5.3 Time course of Induction of NR activity

Figure 4.27 shows the time course of NR activity in cotyledons under continuous RL. The continuous RL increased NR activity in cotyledons of WT, *hp,* and *au* after a lag of 8 h, thereafter NR activity attained a peak at 24 h before declining gradually. At 48 h in WT and *hp,* NR activity was still higher than respective dark controls (Fig. 4.27A) By

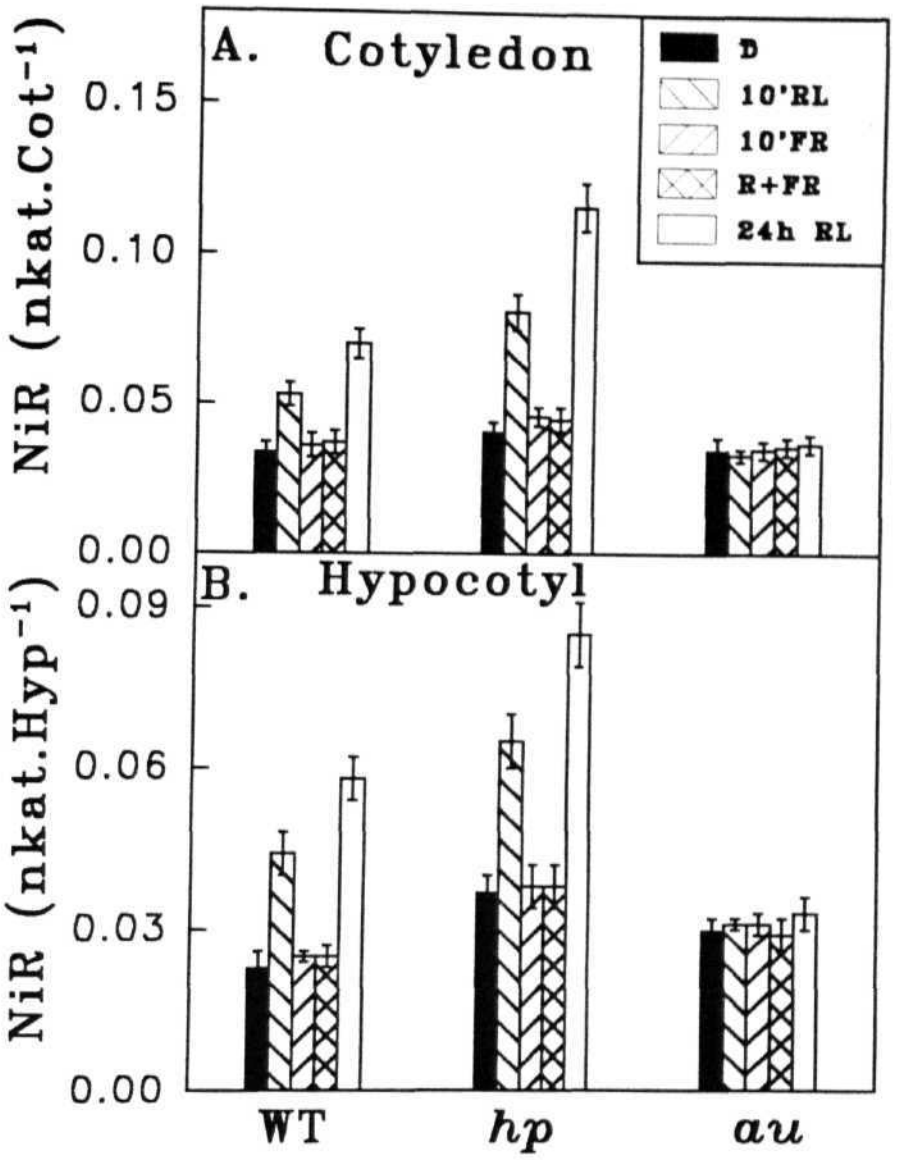


Figure 4.26: Effect of 10 min red light (RL) and far-red (FRL) irradiation on nitrite reductase activity in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown in darkness for 96 h from sowing, and then subjected to continuous RL, brief RL or FRL irradiation as described in the figure. The control seedlings remained in darkness (D). Enzyme activity was estimated at 120 h from sowing.

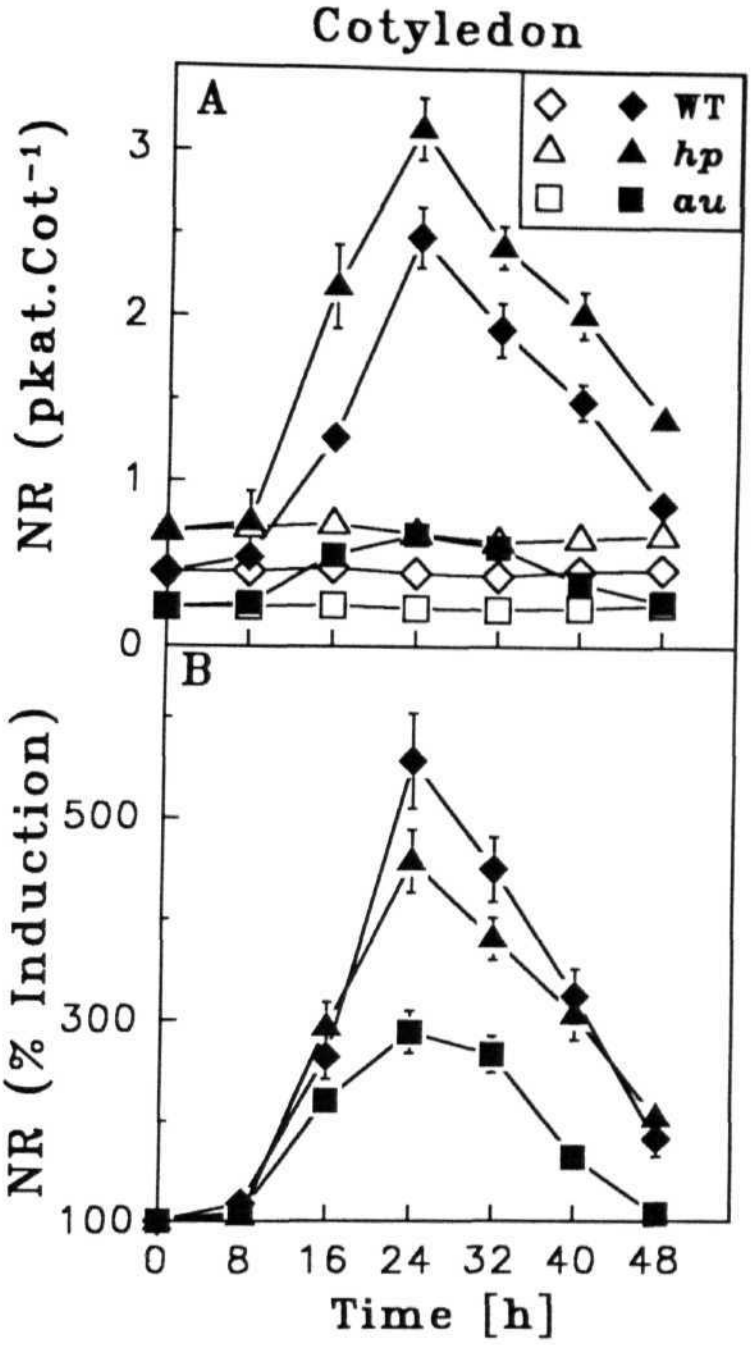


Figure 4.27: (A) Time course of induction of nitrate reductase (NR) activity in cotyledons of tomato seedlings. Seedlings were grown upto 96 h from sowing in darkness, and then transferred to continuous RL (closed symbols). The control seedlings were maintained in darkness (open symbols). (B) The percentage induction in enzyme activity under continuous RL was calculated by taking the enzyme activity in respective dark controls at a given time point as 100%.

contrast, in *au* it was equal to dark control. A comparison of enzyme activities in etiolated seedlings of mutants and WT revealed that in general the basal level of enzyme activities in *au* is less than WT, whereas in *hp* it is more than the WT. Taking into account the observed difference in enzymatic activities of etiolated mutant and WT seedlings, the relative efficiency of mutants and WT to respond to continuous RL, was compared after calculating the percent stimulation of enzyme activity over respective dark controls (Fig. 4.27B). It shows that despite *au* being deficient in phytochrome, retain a significant photo-induction of NR. The time course of NR increases in *au* cotyledons is similar to WT and *hp.* At peak time the photo-induction of NR in *au* is two fold higher than the control whereas, for WT it was 5 fold (Fig. 4.27B). The percent photoinduction of PAL activity in *hp* was slightly less than WT.

In hypocotyl also continuous RL induced NR activity, though the magnitude of induction is nearly 10 times less than that of cotyledons. During the time course of induction, unlike cotyledons, NR activity peaked at 32 h after the onset of RL (Fig. 4.28A). Thereafter, it gradually decreased and by 48 h it reached the dark control level, in all the three genotypes. The percent stimulation of enzyme activity of WT was significantly higher than the *hp* but, since the magnitude of NR activity in *au* hypocotyl is very low ,a higher percent stimulation is observed in this mutant in comparison to WT and *hp* (Fig. 4.28B).

4.5.4 Time course of induction of amylase activity

The time course of amylase activity under continuous RL in cotyledons of mutants and WT (Fig. 4.29A) was similar to that of NR

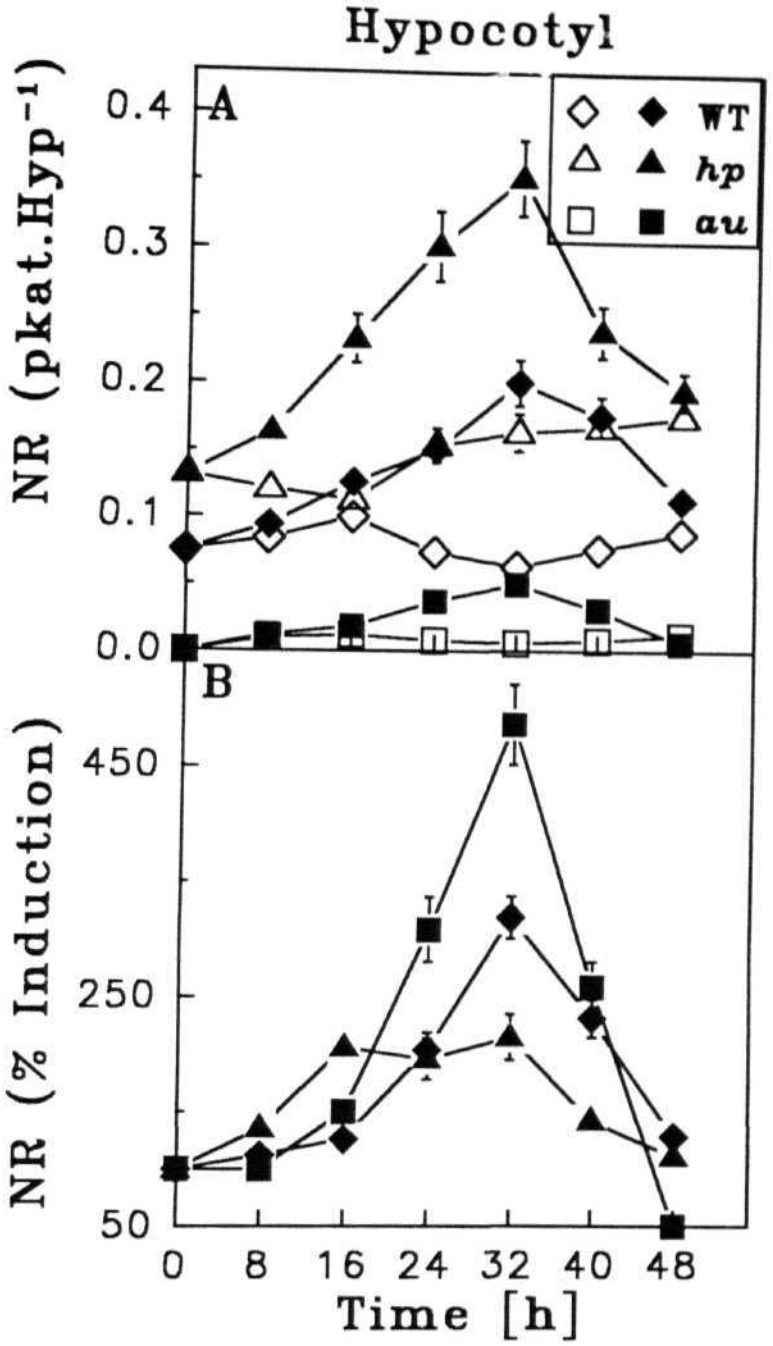


Figure 4.28: (A) Time course of induction of nitrate reductase (NR) activity in hypocotyls of tomato seedlings. Seedlings were grown upto 96 h from sowing in darkness and were then transferred to continuous RL (closed symbols). The control seedlings were maintained in darkness (open symbols). (B) The percentage induction in enzyme activity under continuous RL was calculated by taking the enzyme activity in respective dark controls at a given time point as 100%.

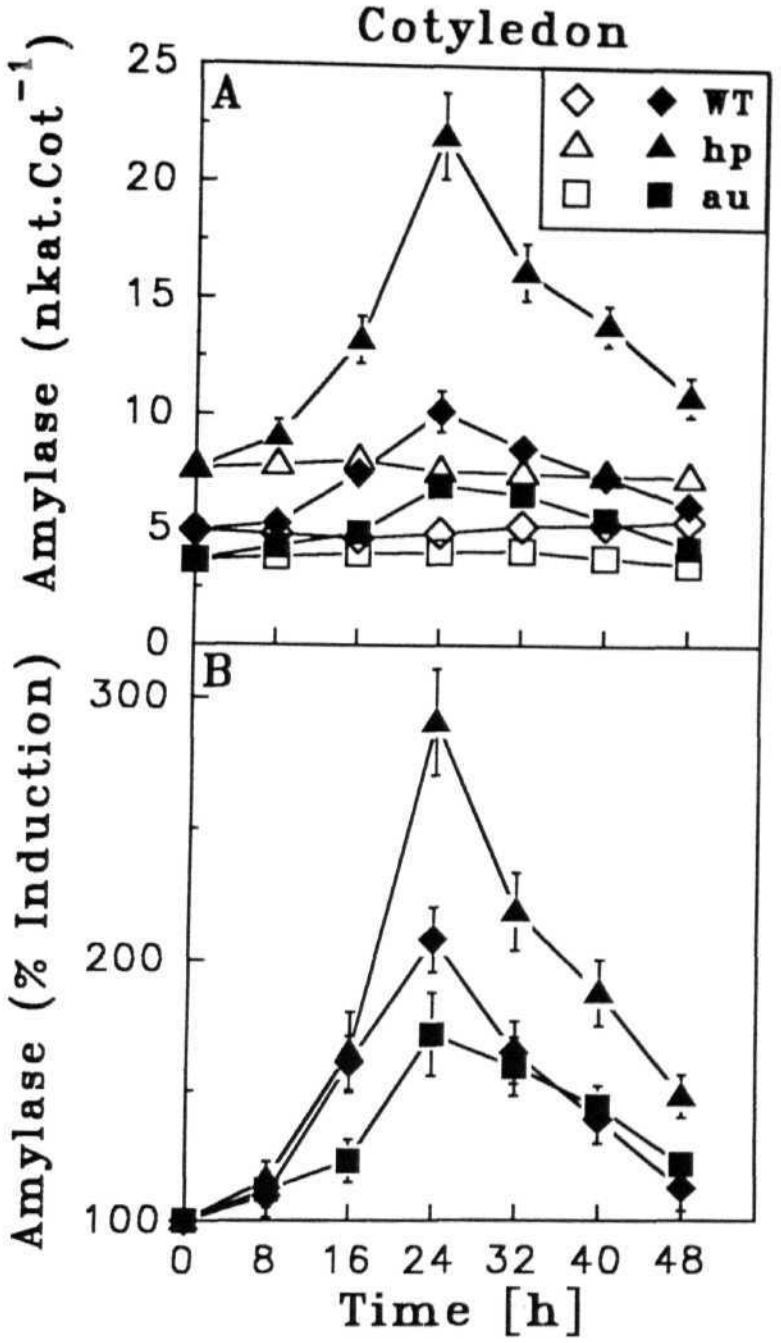


Figure 4.29: (A) Time course of induction of amylase activity in cotyledons of tomato seedlings. Seedlings were grown upto 96 h from sowing in darkness, and then transferred to continuous RL (closed symbols). The control seedlings were maintained in darkness (open symbols). (B) The percentage induction in enzyme activity under continuous RL was calculated by taking the enzyme activity in respective dark controls at a given time point as 100%.

activity in cotyledons (Fig. 4.28). After a initial lag of 8 h amylase activity reached a peak at about 24 h and declined gradually to the level of dark control by 48 h in WT and *au.* Whereas in *hp*, amylase activity was still higher than respective dark control at 48 h. The time course of amylase increase in mutant cotyledons was qualitatively similar to WT, except that induction in *au* at peak time is 66% of WT (Fig. 4.29A). As described under section 4.5.3 the relative efficiency of mutants and WT to respond to continuous RL was compared after calculating the percent stimulation of amylase activity over the respective dark controls (Fig. 4.29B). The relative efficiency of these mutants in inducing amylase activity are in the order of *hp* > WT > *au.*

Unlike NR , the activity levels of amylase in both cotyledons and hypocotyls are similar. The time course of induction of amylase in hypocotyls is very much similar to the time course of cotyledons, where the activity peaks at 24 h (Fig. 4.30A) before declining to dark control level by 48 h in WT and *au* mutant. However, in *hp* at 48 h the amylase activity was higher than the respective dark control. The percent photostimulation profile of hypocotyl amylase induction is very much similar to the profile of cotyledons for both the mutants and WT (Fig. 4.30B).

4.5.5 Time course of induction of nitrite reductase activity

The time course of induction of NiR activity in cotyledons of tomato seedlings was very similar to NR in WT and *hp* seedlings (Fig. 4.31 A). After a initial lag of 8 h, RL induced enhancement of NiR activity reached a peak level at about 24 h, before declining by 48 h, to

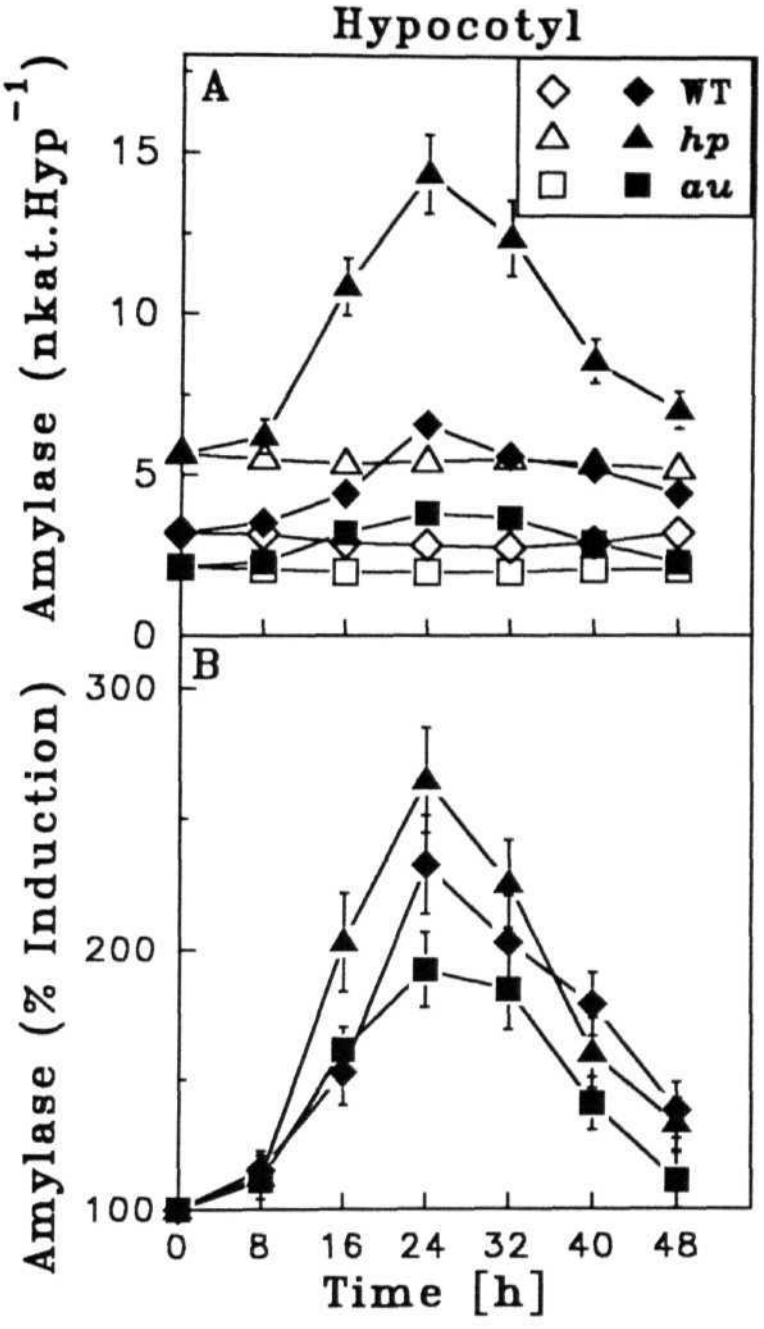


Figure 4.30: (A) Time course of induction of amylase activity in hypocotyls of tomato seedlings. Seedlings were grown upto 96 h from sowing in darkness, and then transferred to continuous RL (closed symbols). The control seedlings were maintained in darkness (open symbols). (B) The percentage induction in enzyme activity under continuous RL was calculated by taking the enzyme activity in respective dark controls at a given time point as 100%.

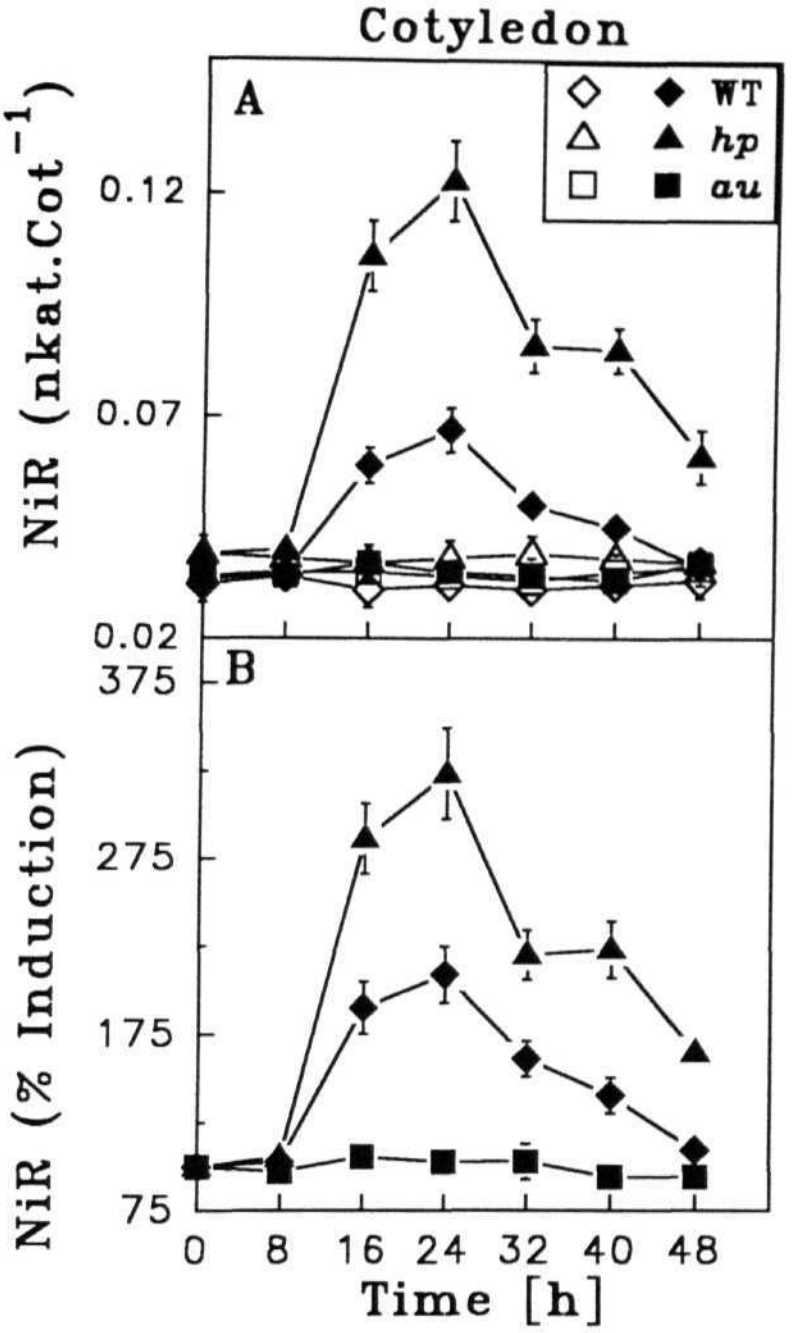


Figure 4.31: (A) Time course of induction of nitrite reductase (NiR) activity in cotyledons of tomato seedlings. Seedlings were grown upto 96 h from sowing in darkness, and then transferred to continuous RL (closed symbols). The control seedlings were maintained in darkness (open symbols). (B) The percentage induction in enzyme activity under continuous RL was calculated by taking the enzyme activity in respective dark controls at a given time point as 100%.

the level of dark control in WT and to a higher level than the respective dark control in *hp*. A higher magnitude of induction of NiR was noticed in cotyledons of *hp* than WT (Fig. 4.31). However, no photoinduction of NiR activity was observed in *au* cotyledons throughout 48 h of continuous RL exposure (Fig. 4.31). The time course of percent stimulation indicates that *hp* mutant is more efficient than WT in photoinduction of NiR activity in cotyledons.

The time course of photoinduction and the Time course of percent induction of NiR activity in hypocotyl is very similar to cotyledons (Fig. 4.32), except that the percent induction of NiR activity is same in both WT and *hp*. There was absolutely no photoinduction of NiR even in the hypocotyl of *au* mutant throughout the 48 h of continuous RL exposure.

4.6 *Effect of BL on NR activity and BL/WL on NiR activity*

Earlier it was reported that BL is required for the normal expression of nuclear encoded plastidic proteins and for the survival of *au* mutant under day light conditions (Oelmüller and Kendrick 1991). In view of this, the effect of BL pretreatment on NR and NiR activity and WL exposure on NiR activity was investigated.

4.6.1 Effect of BL on Nitrate reductase activity

The effect of BL pretreatment on NR activity was investigated in both cotyledons and hypocotyls of tomato seedlings. BL alone showed very little induction of NR activity in cotyledons of tomato seedlings, except in *hp* mutant, where a significant induction of NR activity was

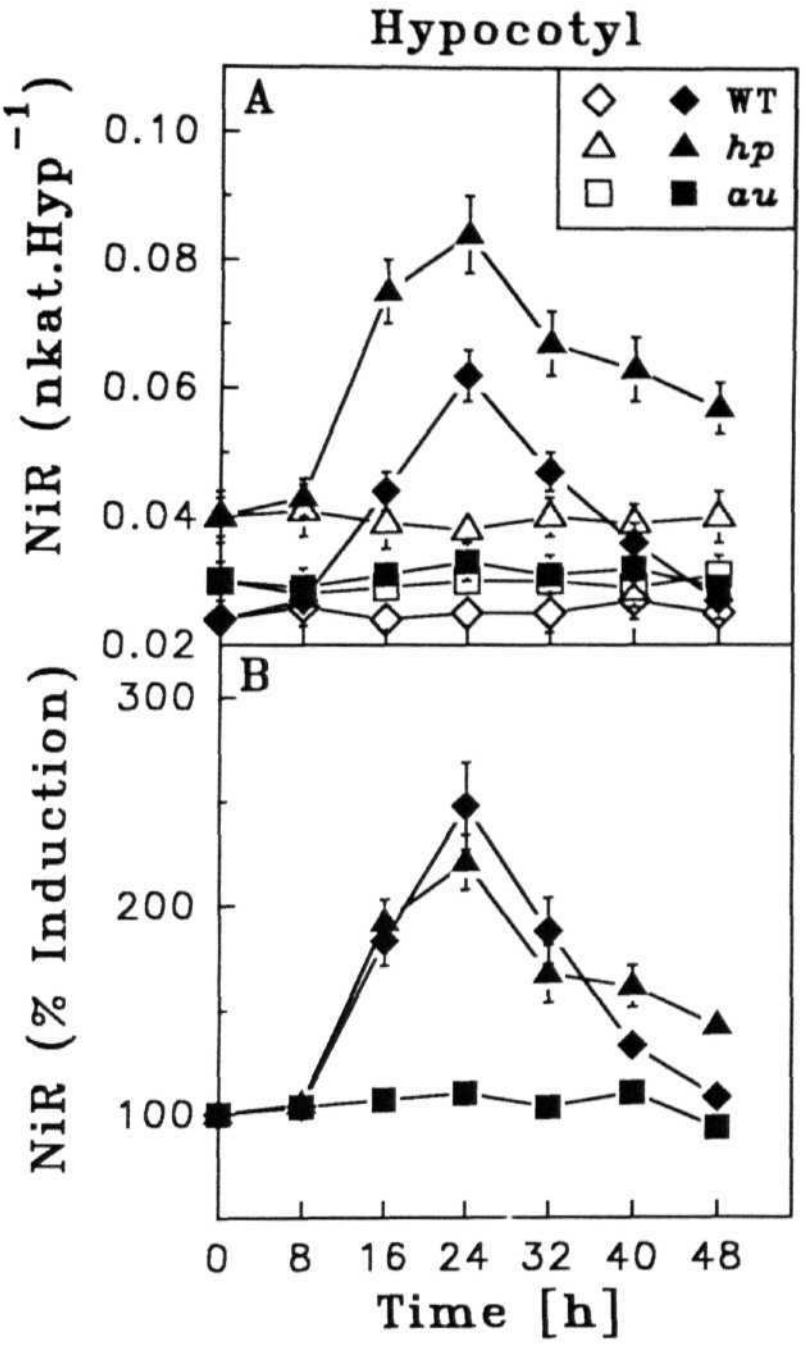


Figure 4.32: (A) Time course of induction of nitrite reductase (NiR) activity in hypocotyls of tomato seedlings. Seedlings were grown upto 96 h from sowing in darkness, and then transferred to continuous RL (closed symbols). The control seedlings were maintained in darkness (open symbols). (B) The percentage induction in enzyme activity under continuous RL was calculated by taking the enzyme activity in respective dark controls at a given time point as 100%.

observed (Fig. 4.33A). There was no significant difference in the photoinduction of NR activity between cotyledons treated with BL before RL and those irradiated with RL alone in both the mutants and WT (Fig. 4.33A). Similarly there was no difference in the induction of NR activity between hypocotyl with BL pretreatment before RL and those irradiated with RL alone (Fig. 4.33B). But, BL alone triggered a very little induction of NR activity even in the hypocotyls of *hp* mutant, along with WT and *au.*

4.6.2 Effect of BL/WL on Nitrite reductase activity

The effect of BL pretreatment on NiR activity was investigated in both cotyledons and hypocotyl of tomato seedlings (Fig. 4.34) In comparison to NR, BL pretreatment induced NiR activity in both cotyledons and hypocotyls of WT and *hp* seedlings and a 12 h BL pretreatment alone was as effective as a 24 h RL treatment in inducing the NiR activity. Moreover, when BL treatment was followed by RL, there was no further increase in the magnitude of NiR induction in both the organs of WT and *hp.* By contrast in *au* mutant there was absolutely no induction of NiR activity with BL, RL or BL followed by RL treatments in both cotyledons (Fig. 4.34A) and hypocotyls (Fig. 4.34B). Whereas, in seedlings transferred to continuous WL, triggered a 60% induction of NiR activity over the respective dark controls in both the organs.

4.7.1 Effect of tungstate on nitrate reductase activity

The contribution of *de novo* synthesis versus activation in photoinduction of NR activity was examined using sodium tungstate.

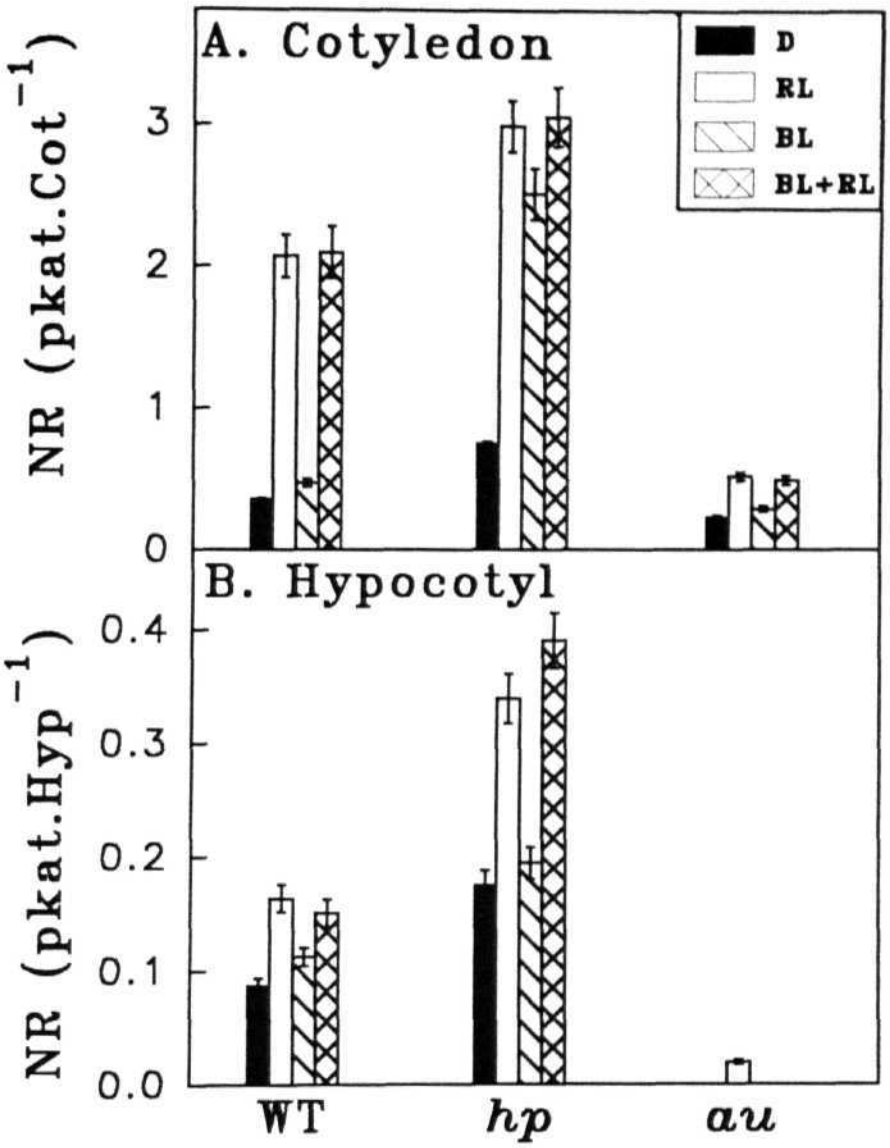


Figure 4.33: Effect of blue light pretreatment on photoinduction of nitrate reductase activity in cotyledons (A) and hypocotyls (B). Seedlings were grown in darkness for 84 h, and then irradiated with BL for 12 h. At the end of blue light treatment, seedlings were either returned to darkness (BL) or were irradiated with continuous RL (RL+BL). In the case of the RL treatment, seedlings were grown upto 96 h from sowing in darkness, and then transferred to the RL. Control seedlings were maintained in continuous darkness (D). Enzyme activity was assayed at 120 h from the time of sowing in all samples.

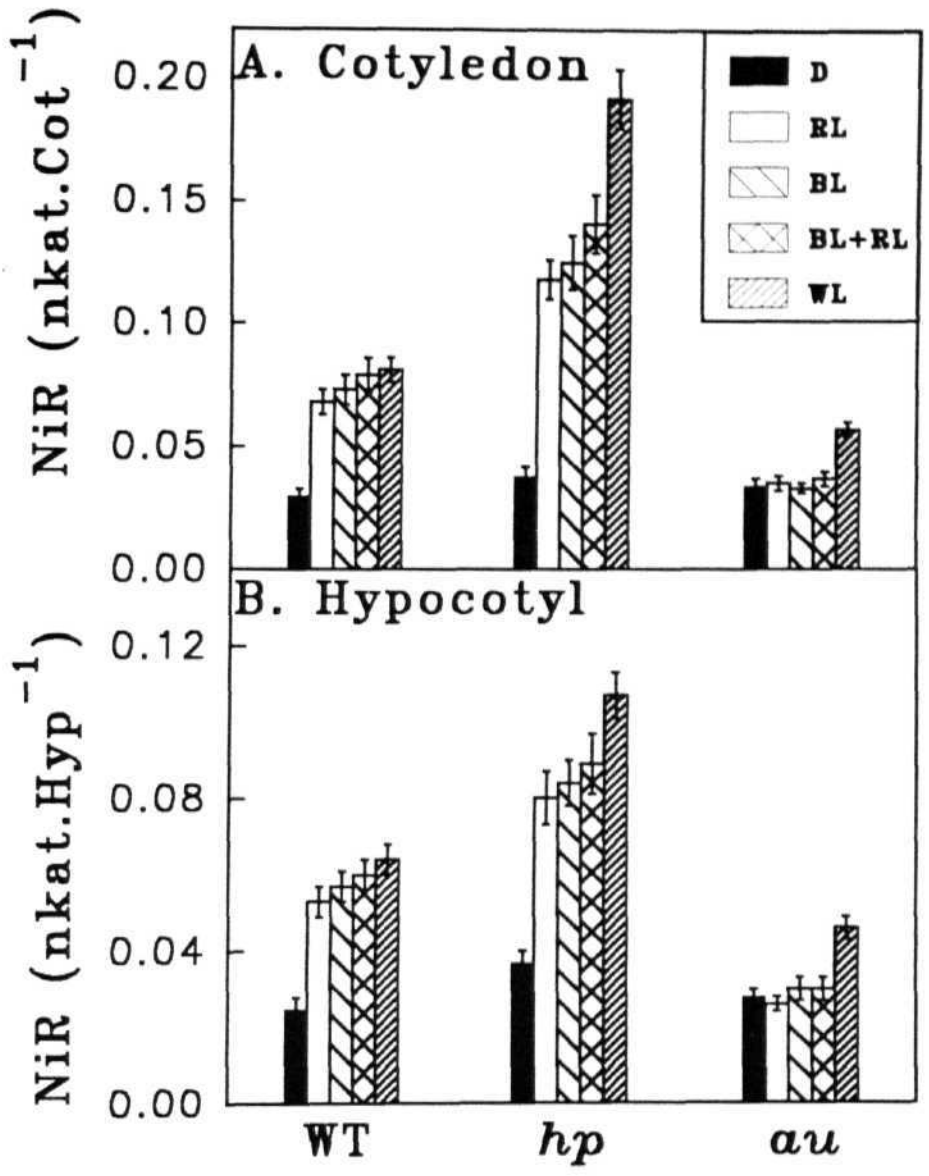


Figure 4.34: Effect of blue light pretreatment on photoinduction of nitrite reductase activity in cotyledons (A) and hypocotyls (B). Seedlings were grown in darkness for 84 h, and then irradiated with BL for 12 h. At the end of blue light treatment, seedlings were either returned to darkness (BL) or were irradiated with continuous RL (RL+BL). In the case of the RL and WL treatments, seedlings were grown upto 96 h from sowing in darkness, and then transferred to the appropriate light. Control seedlings were maintained in continuous darkness (D). Enzyme activity was assayed at 120 h from the time of sowing in all samples.

Tungstate renders newly synthesized NR protein inactive by replacing molybdenum with tungsten as a metal ion (Deng et al., 1989). In seedlings grown in tungstate from the time of sowing, NR activity was less than 5% of both dark and RL grown controls of WT and *hp* cotyledons and no NR activity could be detected in the hypocotyl (Fig. 4.35). Whereas in *au* mutant NR activity was not detectable both in the cotyledons and hypocotyls of seedlings treated with tungstate from the time of sowing.

In the seedlings of mutants and WT, when sprayed with tungstate 2 h before RL treatment (Fig. 4.36), the photoinduction of NR in RL-grown seedlings was totally inhibited. The levels of NR activity in RL grown tungstate treated seedlings were close to dark levels in both cotyledons and hypocotyl.

4.7.2 Effect of loss of nitrate reductase on nitrite reductase activity

Since NR and NiR in higher plants are coordinately induced (Faure et al., 1991) the consequence of loss of NR activity on NiR activity was first examined in tomato seedlings grown on medium containing tungstate from the time of sowing, NiR activity was inhibited by 25% and 40% of dark and RL grown controls respectively in both cotyledons and hypocotyls of WT and *hp* (Fig. 4.37). Whereas, in *au* mutant NiR activity was inhibited by 60% and 80% of dark and RL-grown controls of both the organs.

In seedlings sprayed with tungstate 2 h before RL treatment, the tungstate mediated inhibition of NR activity had no significant effect on

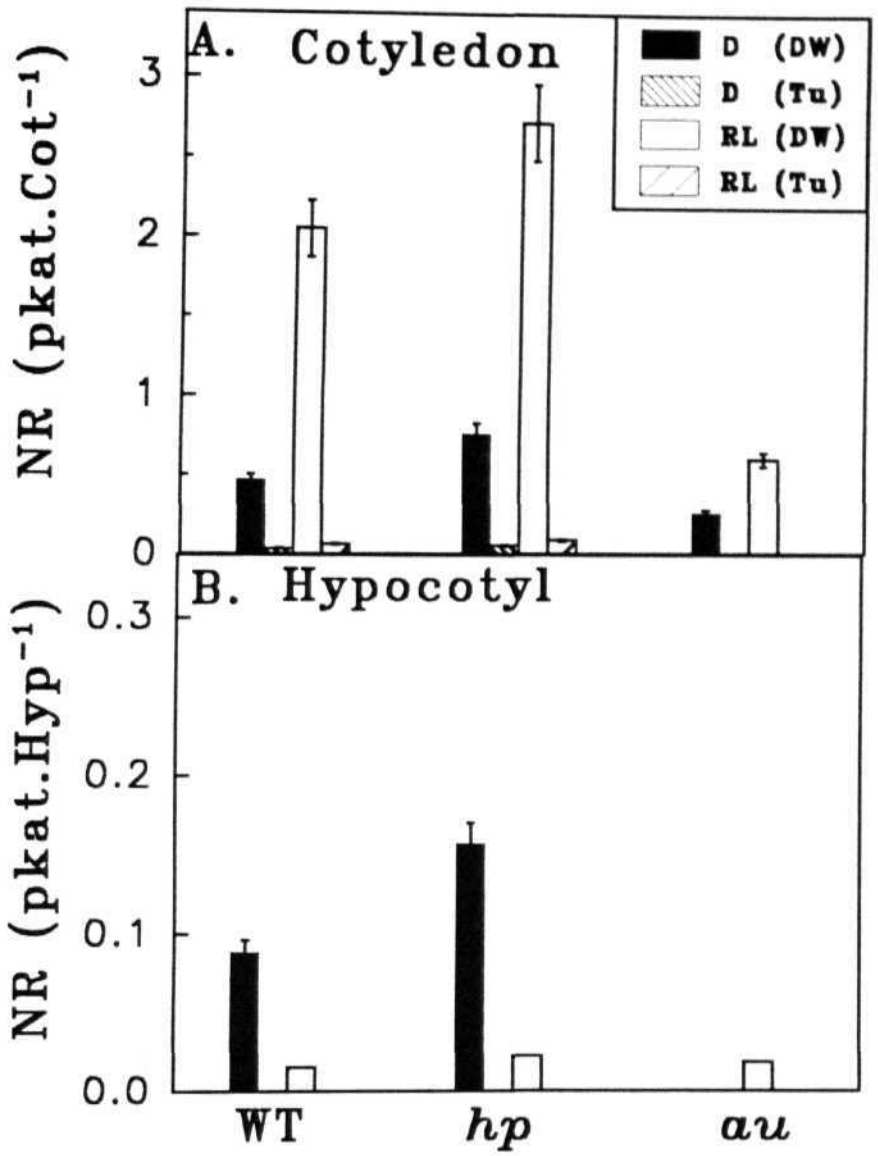


Figure 4.35: Effect of tungstate (Tu) on photoinduction of Nitrate reductase in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown on agar support medium containing tungstate (2 mM) from the time of sowing, whereas controls were grown on medium without tungstate (DW). Seedlings were grown in darkness for 96 h, and then a set of seedlings was transferred to continuous RL. Enzyme activity was measured after 24 h of continuous RL.

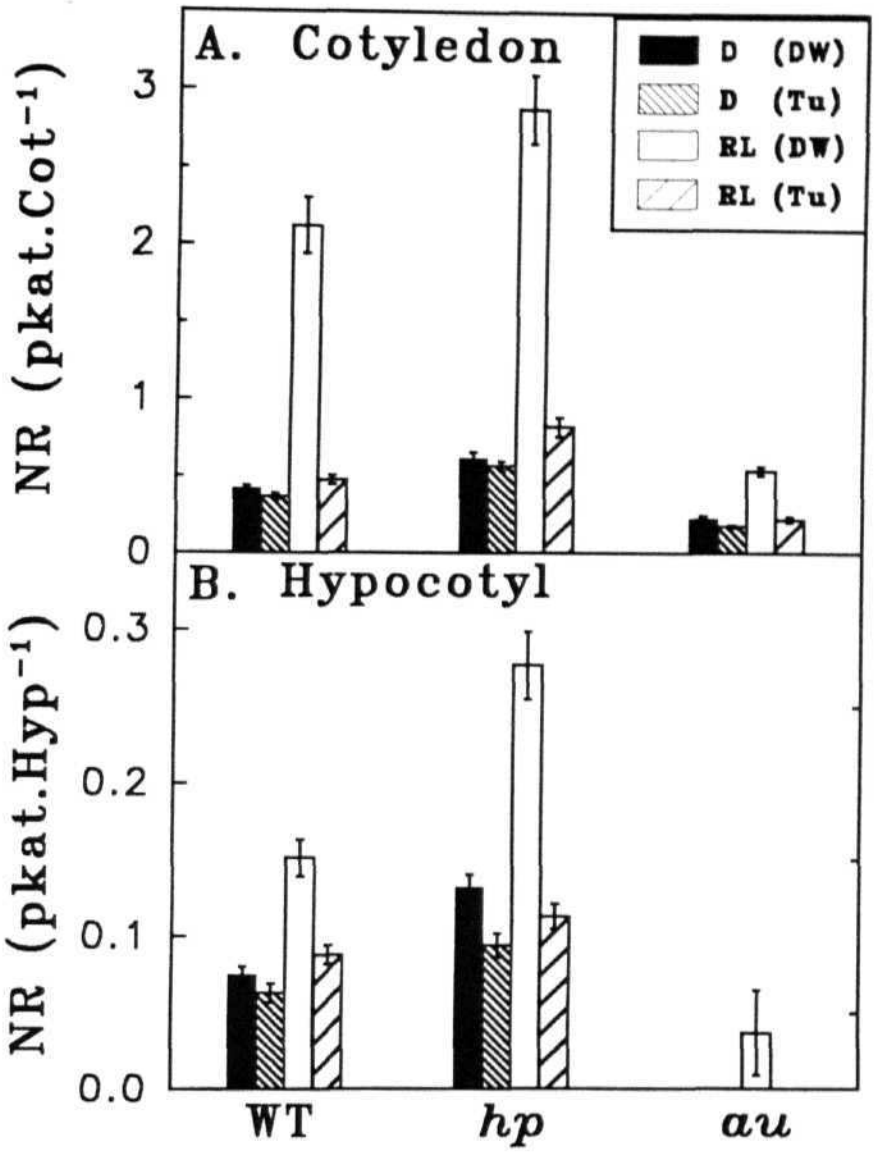


Figure 4.36: Effect of tungstate (Tu) on photoinduction of Nitrate reductase in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown in darkness for 96 h, and then a set of seedlings was transferred to continuous RL. Seedlings were sprayed with tungstate (2 mM) under a green safe light 2 h prior to RL exposure. The control seedlings were similarly sprayed with distilled water (DW). Enzyme activity was measured after 24 h of continuous RL.

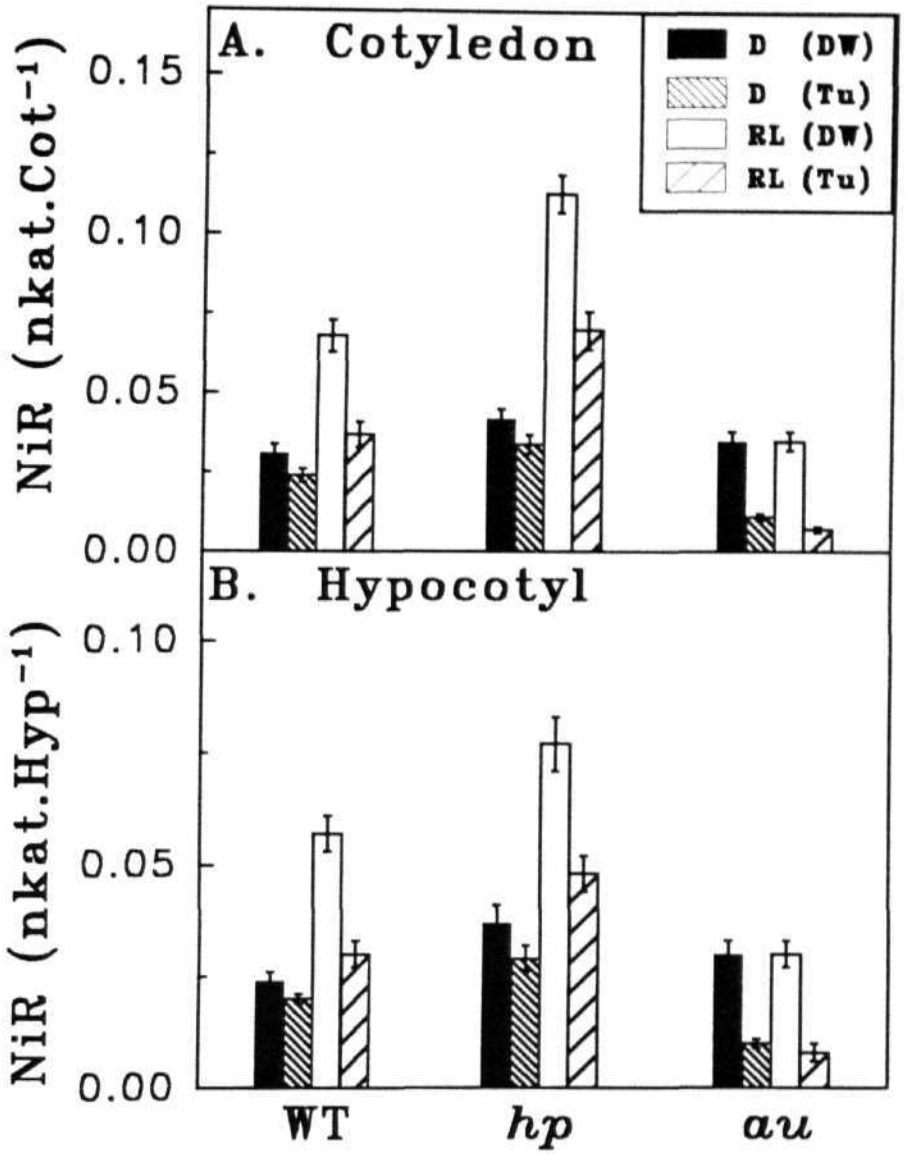


Figure 4.37: Effect of tungstate (Tu) on photoinduction of Nitrite reductase in cotyledons (A) and hypocotyls (B) tomato. Seedlings were grown on agar support medium containing tungstate (2 mM) from the time of sowing, whereas controls were grown on medium without tungstate (DW). Seedlings were grown in darkness for 96 h, and then a set of seedlings was transferred to continuous RL. Enzyme activity was measured after 24 h of continuous RL.

NiR activity in both the cotyledons and hypocotyls of WT and *hp* seedlings (Fig. 4.38) but in me seedlings of *au* mutant, the NiR level dropped below those of the respective controls in both the organs.

4.8.1 Effect of loss of functional plastids on nitrate reductase and amylase activities

In view of the spatial separation of NR and amylase with NiR, their localization being in cytosol and Chloroplast respectively (Rajasekher and Oelmüller 1987). The effect of NF mediated loss of functional plastids (Oelmüller 1989) on NR and amylase activities was studied. In light-grown NF-treated seedlings, NR activity was reduced in cotyledons (Fig. 4.39A) of WT, *hp* and *au* by 25%, 30% and 40% respectively. Whereas, NF-treatment had no effect on the NR activity of dark-grown cotyledons in both the mutants and WT. In hypocotyls (Fig. 4.39B) NF-treatment has reduced the NR activity by 30% in WT and 15% in *hp* in both dark and light-grown seedlings. In *au* hypocotyls there was a complete loss of NR activity due to NF-treatment in both dark and light-grown seedlings. In the case of amylase, NF treatment in dark and light-grown seedlings has very slightly reduced the photoinduction of amylase in both cotyledons and hypocotyl and this reduction was same in both the mutants and WT seedlings (Fig. 4.40).

4.8.2 Effect of loss of functional plastids on Nitrite reductase activity

The effect of NF-mediated loss of functional plastids (Oelmüller 1989) on NiR activity was also studied. In contrast to NR (Fig. 4.39), photoinduction of NiR activity was completely abolished by NF treatment in both cotyledons (Fig. 4.41 A) and hypocotyls (Fig. 4.41B) of

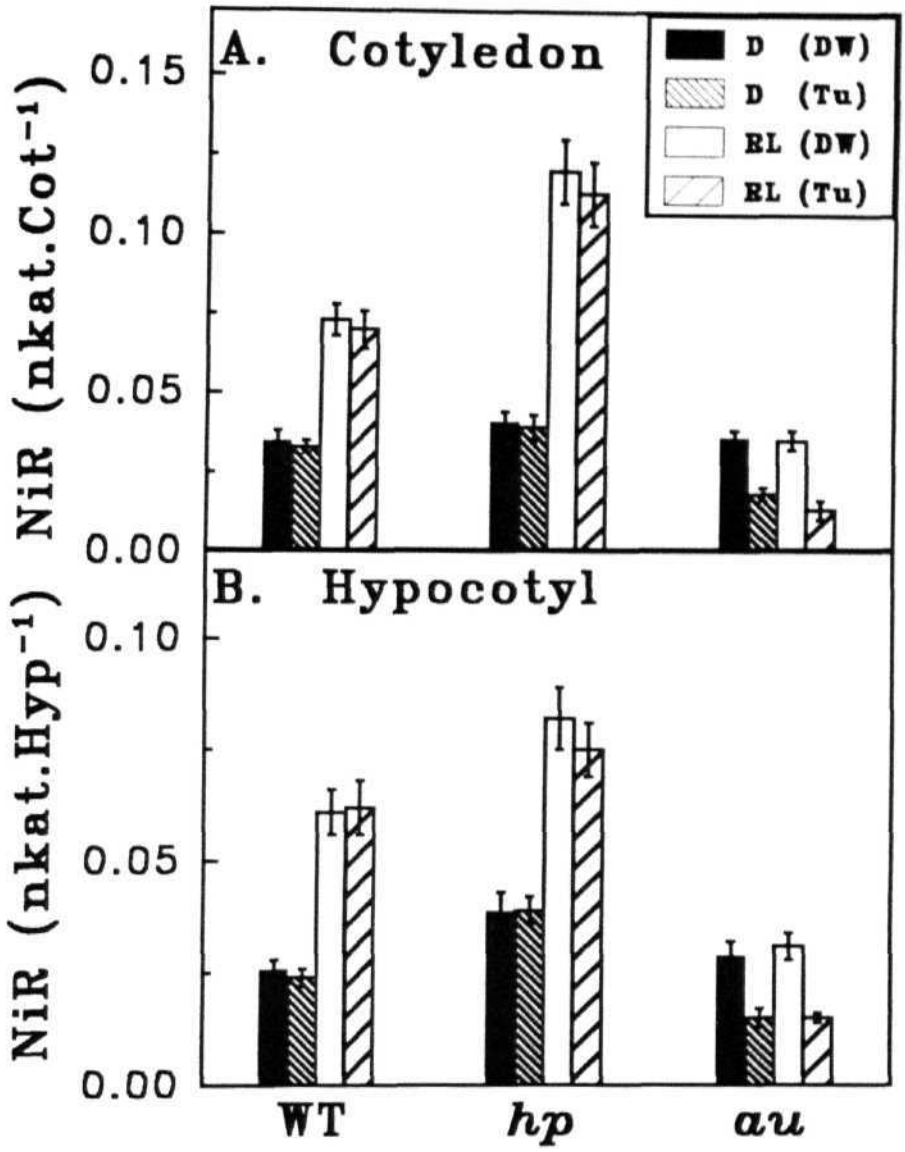


Figure 4.38: Effect of tungstate (Tu) on photoinduction of Nitrite reductase in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown in darkness for 96 h, and then a set of seedlings was transferred to continuous RL. Seedlings were sprayed with tungstate (2 mM) under a green safe light 2 h prior to RL exposure. The control seedlings were similarly sprayed with distilled water (DW). Enzyme activity was measured after 24 h of continuous RL.

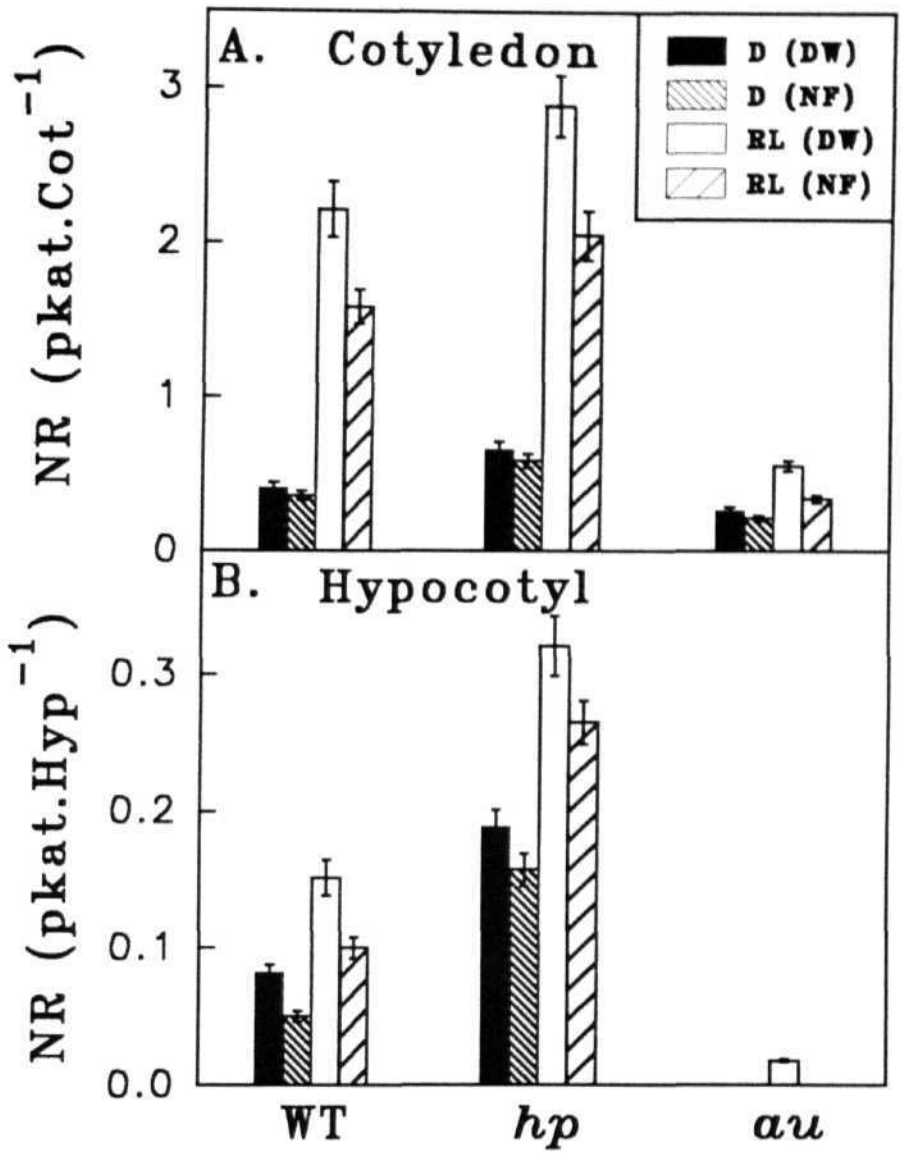


Figure 4.39: Effect of Norflurazon (NF) on photoinduction of NR activity in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown on agar support medium containing NF from the time of sowing, whereas controls were grown on medium without NF (DW). Seedlings were grown in darkness for 96 h, and then a set of seedlings was transferred to continuous RL. The control seedlings were maintained in darkness. Enzyme activity was measured after 24 h of continuous RL.

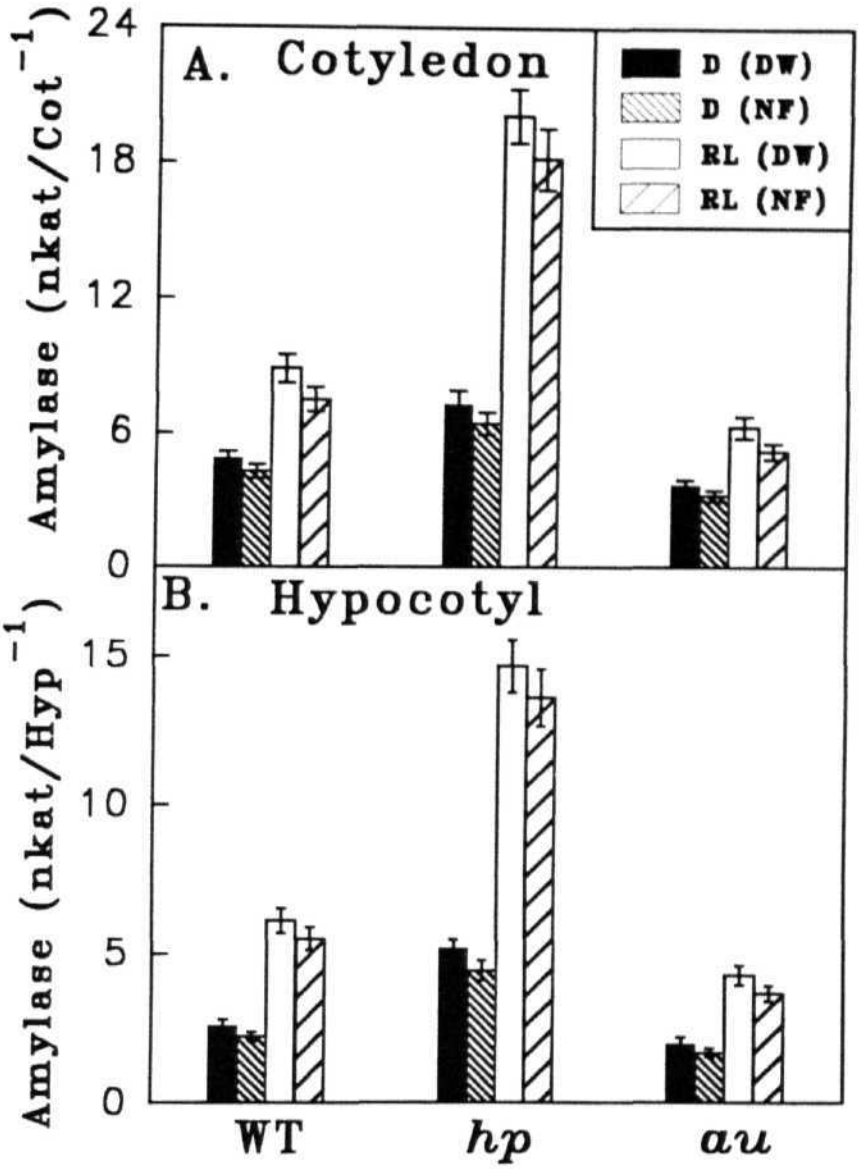


Figure 4.40: Effect of Norflurazon (NF) on photoinduction of amylase activity in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown on agar support medium containing NF from the time of sowing, whereas controls were grown on medium without NF (DW). Seedlings were grown in darkness for 96 h, and then a set of seedlings was transferred to continuous RL. The control seedlings were maintained in darkness. Enzyme activity was measured after 24 h of continuous RL.

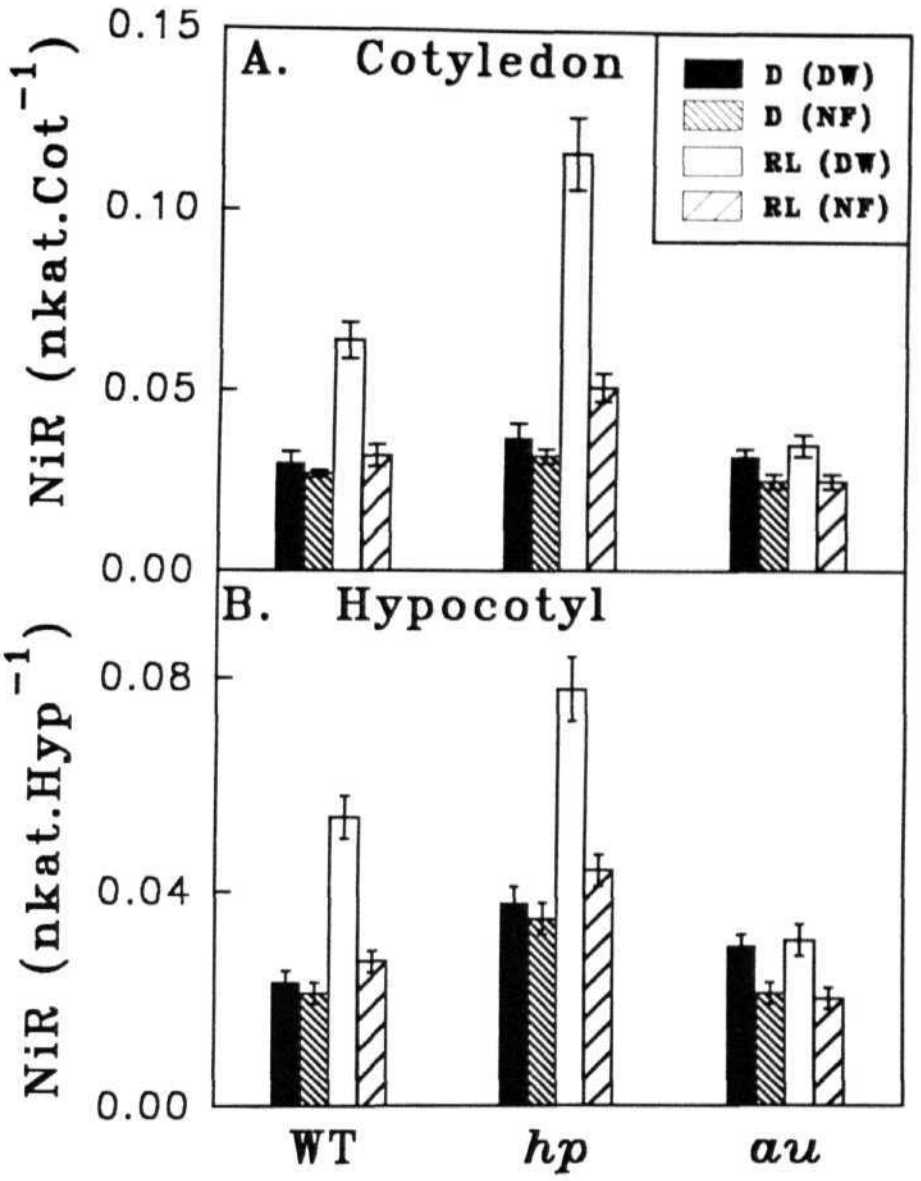


Figure 4.41: Effect of Norflurazon (NF) on photoinduction of NiR activity in cotyledons (A) and hypocotyls (B) of tomato. Seedlings were grown on agar support medium containing NF from the time of sowing, whereas controls were grown on medium without NF (DW). Seedlings were grown in darkness for 96 h, and then a set of seedlings was transferred to continuous RL. The control seedlings were maintained in darkness. Enzyme activity was measured after 24 h of continuous RL.

light-grown WT and the *hp* seedlings. Even in *au* mutant which lacks photoinduction of NiR, the activity level dropped below that of the respective controls in both the organs (Fig. 4.41 A and 4.41B). NF had no effect on NiR activity of dark-grown seedlings in all the three genotypes.

5. DISCUSSION

In spite of the identification and characterization of phytochrome several years ago, its molecular action in controlling photomorphogenesis, and the nature of the putative intermediates in signal transmission have still not been fully deciphered. Among the several approaches currently in use, such as development of transgenics, biochemical analysis of pathways, and use of mutants, the analysis of photomorphogenic mutants has been very fruitful to unravel various hitherto unknown facets of photoreceptor action and signal chain intermediates. In the present study, we employed photomorphogenic mutants of tomato to study the role of phytochrome in regulation of processes like enzyme induction, cell differentiation, cotyledon expansion, and Chloroplast development.

5.1 Phytochrome deficiency and cell differentiation

The most interesting aspect of developmental biology is to know how the cell identifies itself, and differentiates accordingly in response to certain endogenous signals and environmental stimuli. In plants, it is known that multiple stimuli can induce similar developmental responses. For example, several photomorphogenic traits are inducible by various environmental stimuli other than light (Chory et al., 1991, 1994). One of the visible signs of photomorphogenesis in dicot seedlings is that while etiolated seedlings have very few hypocotyl hairs, light-grown seedlings have a very high density of hair formation (Mohr,1972). Similarly,

trichome density on tomato leaves is influenced by both day/night temperatures and photoperiod (Gianfagna et al., 1992).

Molecular genetic analysis has revealed that initiation and expansion of hairs is controlled by no less than 21 genes in *Arabidopsis* of which at least GL1 and TTG gene products are required for initiation. Interestingly, *ttg* mutant which lack hypocotyl hairs have more root hairs than the wild type. Since *au* mutant do possess fewer hairs in dark-grown seedlings, it appear to be defective in controlling the mechanism of hypocotyl hair initiation, and elongation, which appears to be governed by light.

Results from earlier studies clearly demonstrate that the alteration in one developmental pathway may result in defective development of the other pathway. In this study, the lack of RL-stimulated hypocotyl hair development in the phytochrome-deficient *au* mutant may result from deficiency in phytochrome. Since *au* has very few hairs on its hypocotyl, it is likely that a diminished amount of the Pr form of phytochrome may have caused this effect. It has been shown that in *phyB-1* mutant of *Arabidopsis*, absence of phyB leads to abnormal elongation of root hairs in the mutant. It is therefore possible that initiation and elongation of hypocotyl hair in tomato may be mediated by different phytochrome species.

Since *au* possess maximal deficiency of phytochrome in reduction of phyA, the factor responsible for placing of hair in tomato might be under the control of phytochrome A. At the same time, it is also possible that *au* locus is a common point of regulation for both accumulation of

functional phytochrome A and for the expression of the factor responsible for hair density. Since dark-grown seedlings of *au* have fewer hairs when compared to dark-grown WT, it is possible that lack of phytochrome activity in *au* may lead to decline in hair density too. However, it is also likely that such an effect may arise due to the pleiotropic nature of *au* mutation.

The presence of a very high density of hairs on hypocotyl (Fig. 4.5) and accumulation of high amounts of anthocyanin (Table 4.1) in *hp* mutant is analogous to the phenotype of the transgenic *Arabidopsis* mutant which overexpresses the R gene of maize. It is known that anthocyanin induction and hair initiation may be jointly governed by the action of the R and TTG genes. Furthermore, it is reported that the R gene of maize can rescue the *ttg* mutant of *Arabidopsis* which is defective in trichome initiation, by providing TTG function (Lloyd et al., 1992). This may well be the case in WT plants, where the HP gene product may counter the action of the R gene, which is believed to be the universal regulator of anthocyanin induction (Goff et al., 1992). The decline in the level of HP product in *hp* mutant leads to high levels of anthocyanin, and high hair density on hypocotyl. Therefore, the molecular characterization of HP locus in tomato may lead to the isolation of a negative regulator in the photomorphogenic pathway of the above responses.

5.2 Role of phytochrome in cotyledon expansion

Cotyledon expansion in dicots, marked by an increase in size and accumulation of fresh mass, is stimulated by light (Sangeetha and

Sharma 1988). The cotyledon expansion in seedlings with genetically lower levels of phytochromes was severely reduced when compared to WT in continuous RL, indicating a role of phytochrome in cotyledon expansion under these conditions (Neff and Volkenburgh 1994).

In RL-grown *au* seedlings the process of cotyledon expansion was severely inhibited; cotyledons accumulated only a third of the fresh mass of WT. At the same time, *au* cotyledons grew normally under WL. The inefficiency of *au* cotyledon expansion under RL clearly indicates that while residual phytochrome (Sharma et al., 1993) can induce several enzymes in *au* which may be via phyB or other phytochromes, severe deficiency of phyA restricted the cotyledon expansion in it. This is also supported by recent observation in *fri* mutant of tomato which is a null PHYA mutant where an efficient chlorophyll biosynthesis and cotyledon expansion was observed under WL, but not in the seedlings pretreated with FR before transfer to WL (Van Tuinen et al., 1995a) suggesting a role for phyA in cotyledon expansion. This is contrary to the findings in *Arabidopsis* where the light-mediated expansion of cotyledons is attributed to phyB (Neff and Volkenburgh 1994). Similarly, the EODFR response, considered to be a phyB mediated response, is absent in *phyB* mutant of *Arabidopsis (hy3)* (Whitelam and Harberd 1994), but present in *phyB* mutant of tomato (*tri*) (Van Tuinen et al., 1995b). These observations indicate that similar phytochrome forms in different species may perform different roles, depending upon the growth conditions and nature of the surroundings of the plant species.

Studies with BL mutants of *Arabidopsis* have shown that the process of cotyledon expansion is also mediated by BL (Blumm et al., 1994). The restoration of cotyledon expansion in *au* under WL may be as a result of the co-action of the BL-receptor and residual phytochromes. A similar kind of rescue of other photoresponses like induction *of* transcripts of certain plastidic proteins (Oelmuller and Kendrick 1989), induction of NiR activity (Fig. 4.34), chlorophyll accumulation (Fig. 4.7), and cotyledon expansion (Fig. 4.3) was observed in *au* mutant under WL.

5.3 PAL induction in tomato is mediated by a stable pool of phytochrome

Tomato WT seedlings grown under light characteristically accumulate anthocyanin in several regions of seedlings particularly in the hypocotyl hook, and at the junction near the root. However, *au* seedlings completely lack this light-mediated anthocyanin induction. In most systems it is observed that the photoinduction of anthocyanin is preceded by an enhancement in the PAL level. (Beggs et al., 1986, 1987; Brodenfeldt and Mohr 1988; Hahlbrock and Scheel 1989). In view of the causal interrelationship between anthocyanin accumulation and regulation of PAL, we examined the photoregulation of PAL in phytochrome-deficient *au* mutant, hypersensitive *hp* mutant, and in *au,hp* double mutant of tomato.

In tomato, RL initiates a strong photoinduction in PAL activity (Figs. 4.13 and 4.14) and PAL protein (Figs. 4.19 to 4.21) in both the hypocotyl and the cotyledons. This photoinduction of PAL in tomato can

be attributed to phytochrome, since the increase in PAL activity by a brief RL pulse is reversed by a subsequent pulse of FR (Lercari et al., 1982). Interestingly, this photoresponse is observed in all the tomato mutant seedlings, even though the *au* and *au,hp* mutants possess no spectrally active phytochrome in etiolated seedlings (Sharma et al., 1993). The close similarity in the time course of photoinduction of PAL activity in both *au* and *au,hp* mutant to WT indicates that the deficiency in Phytochrome A does not impair the photoregulation of PAL in these mutants. The sustainment of PAL activity at a higher level in the cotyledon and greater magnitude of photoinduction of PAL in the hypocotyl in *hp* mutants are in accordance with it being a signal transduction amplification mutant (Adamse et al., 1988b, Peters et al., 1989). The sustainment of a high level of PAL in cotyledons of *hp* mutants may result from either a reduced rate of PAL degradation or a sustained synthesis of PAL at a steady level. In the *hp* mutant there is a general increase in the PAL level since even in total darkness it is double than that in the WT seedlings.

In seedlings of the *au* genotype the R/FR reversible photoresponses are lacking or severely reduced at the stage of de-etiolation, presumably due to significant reduction in the level of phytochrome A in etiolated seedlings (Sharma et al., 1993). In the present study, it is evident that the FR reversible response on the PAL level in both the hypocotyl and in the cotyledons of *au* and *au,hp* mutant is nearly equivalent to that in WT seedlings. In previous studies with *au* seedlings, such a reversal of an inductive RL pulse by FR has not been observed, except for the photoregulation of CAB gene expression (Sharrock et

al., 1988; Oelmiiller and Kendrick 1991) which is however greatly reduced in magnitude compared with WT. Since *au* is deficient in phytochrome A it is possible that the above PAL response is mediated by the other phytochrome species constituting the stable pool, which has been shown to accumulate in light-grown *au* plants (Adamse et al., 1988a), and which regulates photoresponses in a fashion similar to light-grown WT plants (López-Juez et al., 1990). Out of different phytochrome species constituting the stable pool, at least spectrally active phytochrome B apoprotein levels were shown to be equal to WT (Sharma et al., 1993) and may be involved in PAL induction. But, due to lack of monoclonal antibodies to other forms of phytochromes such as C or D in the stable pool, it is difficult to say which phytochrome species of the stable pool is mediating the observed induction of PAL in *au.* Another possibility, of a low residual phytochrome labile pool present below detection limits mediating PAL induction, can be completely excluded because it was shown that though the level of PHYA polypeptide in etiolated *au* seedlings was about 20% that of WT, it did not show any spectral activity and failed to reconstitute into spectrally active phytochrome in presence of phycocyanobilin both *in vitro* and *in vivo.* The observed R/FR reversible phytochrome response in *au,* similar to the magnitude of WT response, argues against the notion that *au* seedlings are almost red-blind and therefore do not completely de-etiolate under RL.

5.4 Photo-induction of PAL and anthocyanin accumulation are not correlated

The photoinduction of PAL in tomato seedlings does not show good correlation with stimulation of anthocyanin biosynthesis in the same seedlings (Adamse et al., 1989). In the *au* and *au,hp* seedlings, little anthocyanin is produced, but the time course of increase in PAL level in respect to RL is similar to WT. Similarly, in the hypocotyl, a correlation between PAL induction and anthocyanin level is not evident. In tomato seedlings, the majority of anthocyanin biosynthesis takes place in the hypocotyl and its decrease is completed within 8 h, before the onset of the sustained increase in the anthocyanin level (Peters et al., 1991). Although RL irradiation leads to a greater enhancement in the level of PAL in the hypocotyl of *hp* mutants, PAL activity is also significantly enhanced in WT, *au*, and *au,hp* mutant seedlings which fail to produce much anthocyanin (Table 4.1). Furthermore, in *au* seedlings a single pulse of RL significantly increases PAL level, but the same *au* seedlings do not show any detectable level of anthocyanin with a single RL pulse (Adamse et al., 1989). Moreover, in all the genotypes used except the *hp* mutant, a blue light pretreatment is required to observe the phytochrome regulation of anthocyanin induction (Adamse 1988; Mancinelli 1985) while in the present study the genotypes used exhibited R/FR reversible photoregulation of PAL without a blue light pretreatment.

It is evident from the foregoing discussion that there is no strict correlation between the photoinduction of PAL measured here, and the biosynthesis of anthocyanin in tomato seedlings. The lack of correlation between photoinduction of anthocyanin biosynthesis and PAL has been

noticed in other systems on the basis of lack of coordination in the kinetics of photoinduction of PAL and anthocyanin accumulation (Brodenfeldt and Mohr 1988), while our studies rule out a direct correlation between a phytochrome-mediated induction of the major pool of PAL and anthocyanin biosynthesis. But, they do not rule out a key role played by PAL in anthocyanin biosynthetic pathway, since the inhibition of PAL activity *in vivo* by α-aminoxy, β-phenyl propionic acid, strongly reduces the photoinduction of anthocyanin in tomato seedlings (Table 4.1).

PAL activity is a prerequisite for anthocyanin synthesis. In mustard seedlings where anthocyanin is localized in the lower epidermis of cotyledons, after dissecting cotyledons into lower and upper parts, Beggs et al. (1987) found a correlation between PAL and anthocyanin increase in the lower epidermis during the first few hours of anthocyanin accumulation. Since anthocyanin in tomato seedlings is also strictly localized in the lower epidermal layer of the cotyledons and the sub-epidermal layer of the hypocotyl, it is possible that PAL activity in these epidermal layers follows a different kinetic pattern of photoregulation than the total pool in the whole organs. The inhibition of anthocyanin accumulation by a-aminoxy, p-phenyl propionic acid (Table 4.1) indicates that a minor pool of PAL may play a role in anthocyanin accumulation. However, such tissue-specific correlation between photoregulation of PAL and end product accumulation has not been firmly established for PAL. Another enzyme, chalcone synthase, which plays an important role in regulating anthocyanin synthesis, clearly

shows a tissue specific distribution and induction by light (Ehmann et al., 1991; Schmelzer et al., 1988).

The inhibition of PAL photoinduction by RNA and protein synthesis inhibitors (Figs. 4.17 and 4.18) indicates that PAL is synthesized *de novo* in tomato seedlings (Brodenfeldt and Mohr 1986). This was further confirmed by western blotting using tobacco PAL antibodies, which showed the accumulation of PAL protein till 6 h of irradiation in *au* (Fig. 4.20) and WT (Fig. 4.19), whereas in *hp* (Fig. 4.21), under RL, higher PAL levels were observed till 12 h of irradiation and decreased by 24 h in cotyledons and hypocotyls of all the mutants and WT seedlings.

From the observations of the present study, it is evident that although phytochrome mediates PAL induction, the major pool of PAL present in tomato seedlings does not participate in controlling the anthocyanin level upon exposure of etiolated seedlings to continuous RL. The results presented in this study also indicate that while the photoregulation of anthocyanin synthesis is dependent on phyA and the photoregulation of PAL is proposed to be mediated by the stable pool in tomato seedlings. But, on contrary to this the PhyB deficient *tri* mutant of tomato also accumulates less amounts of anthocyanin in seedlings grown under RL suggesting a role for PhyB also.

This paradox can be resolved by studying the PAL induction and anthocyanin biosynthesis in a proven PHYA and PHYB null mutants like *fri* (Van Tuinen et al., 1995a) and *tri* (Van Tuinen et al., 1995b) mutants along with the doubly null mutants of PHYA and PHYB. These studies

will also help in identification of certain exclusive and some overlapping functions of both PHYA and PHYB.

5.5 A urea mutant retains photoinduction of cytosolic enzymes, but lacks photoinduction of plastidic enzymes

Earlier studies on RL-mediated photoinduction of several nuclear encoded plastidic genes revealed that *au* seedlings lack photoinduction of plastidic proteins and need blue light to elicit Pfr action on these proteins; but under white light, *au* plants survive and complete their life cycle normally (Oelmüller and Kendrick 1991). By contrast, our results indicate that even brief pulse of RL can induce PAL activity in *au* seedlings. Since PAL is a cytosolic enzyme, it would be quite logical and interesting to know whether other cytosolic enzymes are also similarly induced by RL in *au* and nature of the RL-mediated photoinduction of plastidic enzymes in *au.*

The results obtained with *au* in this study are in accordance with the fact that the level of physiologically functional phytochrome is severely reduced in etiolated seedlings of this mutant. Although the dark-grown seedlings of *au* possessed a basal level of amylase (Fig. 4.23), NR (Fig. 4.24), and NiR (Fig. 4.25) activity, a brief RL pulse did not stimulate any of these enzymes in *au*, but stimulation was observed in WT and *hp* which was reversible with FR pulse. The absence of RL pulse-mediated enzyme induction in *au* is consistent with previous studies, demonstrating that *au* shows little induction of photoresponses with RL pulses (Oelmüller et al., 1989; Oelmüller and Kendrick 1991; Becker et al., 1992). Since PAL in *au* can be induced by brief RL pulses,

and NR and NiR do not respond to the same pulse, it is evident that different enzyme inductions require different amounts or fluence to initiate response, PAL being more sensitive than NR and NiR. Although *au* did not respond to brief RL pulses, it showed a stimulation of amylases and NR activity under continuous RL, in a fashion qualitatively similar to WT and *hp* seedlings. This stimulation can also be attributed to phytochrome because in addition to R/FR reversible low-energy response, the continuous RL mediated high-irradiance response also represents a criterion for the involvement of phytochrome, and in case of *au,* it may be one or more of the stable pool phytochrome species.

The time course of NR (Figs. 4.26 and 4.27) and amylase (Figs. 4.28 and 4.29) induction in *au* under continuous RL followed profiles similar to those in WT and *hp,* except that the magnitude of enzyme induction in *au* was considerably lower than in WT and *hp.* Evidently, barring the absence of NiR photoinduction in *au,* the deficiency of phytochrome in *au* or amplification of sensitivity to phytochrome in *hp* did not influence the profiles of enzyme induction, such as the duration of lag or the time required to attain peak induction of enzymes. The higher magnitude of photoinduction of enzymes in *hp* seedlings are in conformity with the observed pleiotropic effect of *hp* mutation on amplification of phytochrome-regulated responses (Peters et al., 1992). By contrast, the observation that continuous RL stimulates amylase, NR, and PAL activities in *au,* is at variance with previous studies where continuous RL-mediated induction of several nuclear transcripts could not be detected in etiolated *au* seedlings (Sharrock et al., 1988; Oelmüller et al., 1989; Oelmüller and Kendrick 1991).

The retention of continuous RL-mediated induction of NR and amylase along with PAL in seedlings, despite severe reduction in the spectrally active phytochrome A level, indicates that *au* has a residual active phytochrome pool that regulates the above responses. Physiological experiments have shown that mature plants *of au* retain the EODFR response (Adamse et al., 1988; López-Juez et al., 1990) and shade-avoidance reactions (Whitelam and Smith 1991; Kerckhoffs et al., 1992) which are assumed to be mediated by a photostable phytochrome. Since mutants deficient in PHYB apoprotein (López-Juez et al., 1992; Reed et al., 1992, 1993) lack the above responses, it is assumed that at least PHYB is functional in mature *au* plants. This was biochemically confirmed by showing that the elution profile of spectrally active phytochrome from green leave of *au* was similar to that of PHYB, indicating PHYB is spectrally active (Sharma et al., 1993). Considering the existence of spectrally active phytochrome in green *au* plants and the retention of phytochrome triggered responses in *au* seedlings, it is plausible that the residual phytochrome pool of *au* seedlings may consists of photostable phytochrome species. However, as discussed, under the PAL induction the relative proportion and functional contributions of phytochrome species constituting the stable phytochrome pool in *au* are not known because of the lack of monoclonal antibodies to other individual phytochrome species.

5.6 Chloroplast development in aurea mutant

Although it has been tacitly assumed that defective photo-regulation in *au* arises from phytochrome deficiency, the pleiotropic

nature of the *au* mutation cannot be ignored. Though it has been suggested that *au* is a PHYA deficient mutant (Sharma et al., 1993); Parks and Quail 1993; Whitelam et al., 1993), *au* plants grown under normal day light differ from WT, being pale green in color (López-Juez et al., 1990; Becker et al., 1992) In comparison, the phenotype *of PHYA* null mutants of both *Arabidopsis* (Nagatani et al., 1993) and tomato (Van Tuinen et al., 1995a) are indistinguishable from WT. Unlike WT, *au* possess a agranal Chloroplast with a reduced number of thylakoid membranes (Koornneef et al., 1985). Such a pleiotropic effect of *au* mutation on Chloroplast development indicates that in addition to phytochrome deficiency, the lack of a functional Chloroplast, or defective Chloroplast may also influence expression of nuclear encoded genes like NiR (Oelmüller et al., 1989). For instance, it has been shown that expression of nuclear genes that encode plastidic proteins like CAB and RBCS is closely associated with Chloroplast development (Susek et al., 1993). In the present study, although photoinduction of cytosolic enzymes such as amylase and NR are present in *au*, a similar induction of plastidic proteins like NiR and of mRNA levels for other plastidic proteins (Sharrock et al., 198S: Oelmüller and Kendrick 1991) is not seen. The absence of photoinduction of NiR in *au*, even under continuous RL, is an intriguing observation because continuous RL stimulated accumulation of NR and NiR transcripts. Becker et al. (1992) showed that although a brief RL pulse failed to elevate the NiR and NR transcript levels in *au* continuous RL elevated both NR and NiR transcripts. In contrast, RL mediated increase in NR transcript level (Becker et al., 1992), and stimulated *de novo* synthesis of NR in *au* (Figs. 4.34 to 4.37) as revealed by tungstate mediated inactivation of newly

synthesized NR molecules (Deng et al., 1989); a similar increase in NiR transcript level in *au* is not accompanied by a stimulation in NiR enzyme level.

It is likely that the observed discrepancy between the RL-mediated increase in NiR transcript level and the absence of photoinduction of NiR activity may arise from a block in Chloroplast development in *au.* Although phytochrome induction of NiR transcript (Becker et al., 1992) may not be tightly linked with Chloroplast differentiation, but because it is a plastidic enzyme, the expression of NiR activity is likely to be dependent on Chloroplast development. Neuhaus et al. (1993) has unequivocally shown that in hypocotyl cells of etiolated *au* seedlings, plastid development is arrested at the level of proplastids which do not even differentiate into etioplasts. Moreover, these proplastids do not transform to chloroplasts even after a 48 h exposure to WL. In the present study also, it was demonstrated that cotyledons of *au* seedlings possess a delayed and sluggish acquisition of photosynthetic oxygen evolution by monitoring the capacity of chloroplasts to acquire *in vivo* light-mediated carbon dioxide dependent oxygen evolution. This also suggested that the *au* mutant is impaired in photoinduction of Chloroplast differentiation. It is possible that the above delay in Chloroplast development in *au* may in some way be responsible for the absence of photoinduction of NiR. The observation that NF induced photo-oxidation of chloroplasts, drastically reduces the NiR levels in wild type and *hp* indicating that the chloroplast's integrity is essential for photostimulation of NiR activity (Oelmüller et al., 1989). The above discussion highlights the fact that even though *au* retains

photinduction of enzymes that are possibly regulated by residual phytochrome, deficiency in NiR photoinduction may be either due to PHYA deficiency or due to Chloroplast development.

5.7 Role of BL in au development

Since *au* mutants survive despite being deficient in phytochrome and having impaired Chloroplast biogenesis, it is possible that co-action of another photoreceptor during de-etiolation may alleviate the adverse affects of phytochrome deficiency. For example, BL pretreatment of *au* restores phytochrome mediated induction of nuclear transcripts encoding plastidic proteins (Oelmüller and Kendrick 1991). Although NiR is a nuclear-encoded plastidic protein, a BL pretreatment of *au* did not induce NiR activity, whereas BL largely replaced RL-mediated NiR induction in *hp* and WT . Only when *au* seedlings were exposed to continuous WL could a reduced level of NiR photoinduction be observed. likewise photoinduction of RBCS transcripts in *au* was seen only under continuous WL in *au* (Sharrock et al., 1988). Probably a simultaneous operation of BL photoreceptor and residual phytochrome under continuous WL restores photoinduction of NiR by stimulating Chloroplast development in WL seedlings.

5.8 General conclusions

The physiological studies so far carried out in *au* mutant revealed several abnormalities right from the process of germination to the completion of its life cycle, but the actual reason for the observed deficiencies is still not precisely known. At the seedling stage *au* mutant

exhibit more pleiotropic phenotype compared to WT due to phytochrome deficiency which is characterized by reduction in

(i) hypocotyl growth inhibition in WL, FR, R, B, and UV-A (Koornneef et al., 1985; Adamse et al., 1988),

(ii) Chlorophyll and Chloroplast development (Koornneef et al., 1985; Ken Dror and Horwitz 1990; Neuhaus et al., 1993)

(iii) Anthocyanin content (Adamse et al., 1989)

(iv) The photoregulation of the transcript levels of chlorophyll a/b binding proteins of PSI and PSII, plastocyanin and subunit II of photosystem 1 (Sharrock et al., 1988; Oelmüller and Kendrick 1991).

Adding to this list, the present work has identified

(i) the reduced photoinduction of cytosolic enzymes like PAL, NR, amylase

(ii) total loss of photoinduction of plastidic enzyme NiR

(iii) reduced cotyledon expansion, chlorophyll accumulation and photosynthetic oxygen evolution under RL, and

(iv) presence of short and low density of hairs on hypocotyl.

Analysis of the results obtained earlier and the present data suggest that in *au* mutant, phytochrome deficiency is targeted more towards phyA than other phytochromes. However, the recent isolation and characterization of type-specific *phyA* and *phyB* mutants in tomato led to some contrasting suggestions. For example, the phyA deficient *fri* mutant was selectively insensitive to FR (Van Tuinen et al., 1995a) and phyB deficient *tri* mutant is insensitive to RL alone (Van Tuinen et al., 1995b) whereas, the lack of responsiveness of the *au* mutant to both R and FR is pointing it towards being a chromophore mutant. At the same time,

earlier evidence such as presence of reduced levels of immunochemically detectable phyA, WT levels of spectrophotometrically detectable phytochrome, and immunochemically detectable phyB (Sharma et al., 1993) in *au* mutant and their absence in well established chromophore mutants strongly suggest that *au* is not a true chromophore mutant. Both phyA and phyBl deficient mutants of tomato exhibited normal EODFR responses, indicating that either phyB2 or other phytochrome forms mediate this response. The presence of

(i) normal EODFR response (López-Juez et al., 1990)

(ii) normal response to very high reduction in R:FR photo ratio (Kerckhoffs et al., 1992) though less sensitive response to very low changes in R:FR ratio than WT (Casal and Kendrick 1993), and

(iii) R/FR reversible induction of PAL activity

in *au* mutant indicate that *au* does have functional phytochrome species which cannot be expected from a true chromophore mutant. Moreover, the exogenous supply of chromophore could restore the WT phenotype in all the chromophore mutants of *Arabidopsis* (Parks and Quail 1991) and tobacco *pew 1-1* mutant (Kraepiel et al., 1994). In contrast, in *au* mutant, both *in vivo* and *in vitro* attempts to rescue *au* phenotype and *au* polypeptide by exogenous supply of chromophore were unsuccessful (Reddy and Sharma, personal communication), as in the case of *pew2-l* mutant of tobacco.

The *pew2-l* mutant of tobacco to a great extent is similar to *au* mutant of tomato: first, it does not show rescue by exogenous biliveridin, second, it produces reduced levels of phyA polypeptide, and third, it has a normal level of light-stable phytochrome when grown under WL. In

contrast, *pew1-1* mutant, which is a true chromophore mutant, possess normal levels of phytochrome apoprotein **in dark and in** light-grown plants but the level of spectrally active phytochrome is severely reduced. Taking these data together we suggest that since tobacco and tomato belong to same solanaceae family, both *pew2-1* locus and *au* locus may have something in common, and may represent a mutation distinct from normal chromophore mutants.

Though it may be debatable whether *au* is a true *phyA* deficient mutant or not, Neuhaus et al. (1993) presented evidence that microinjection of purified phytochrome A into hypocotyl cells of *au* mutant complements the *au* phenotype by inducing anthocyanin accumulation and Chloroplast development. It is suggested that though *au* is not a null phytochrome mutant, the roles of PhyA deduced from the physiological analysis *of au* can be assigned to phyA and the signaling intermediates identified by its biochemical analysis can be considered part of the phyA signal-transduction pathway. From our observation we speculate that reduction in the level of PHYA polypeptide in *au* may be because of a defect in a more general process such as translation, or a defect in some post-translational modification step specific for phyA leading to the lack of bilin C-S lyase activity and increased degradation of the protein.

Despite the deficiencies such as

(i) 30-40% decreased chlorophyll and RuBPCase content (Becker et al., 1992),

(ii) reduction in the number of thylakoids in Chloroplast (Koornneef et a!., 1985; Neuhaus et al., 1993),

(iii) deficiency of protochlorophyllide in dark grown seedlings (Ken-Dror and Horwitz 1990), and

(iv) being defective in both abundance and light regulation of light-harvesting chlorophyll a/b binding polypeptides (Ken-Dror and Horwitz 1990),

the maximum photosynthetic rate of the *au* mutant is only slightly reduced. The *au* mutation does not result in damage or marked loss of efficiency in the photosynthetic electron-transport system under continuous WL (Becker et al., 1992). However, the net leaf photosynthesis under vegetation shade is significantly affected by the phytochrome deficiency but the *au* lesion has little effect on the capacity of tomato to exhibit photoadaptation to stimulated vegetation shade when measured at the thylakoid level (Smith et al., 1993). The comparison of the photosynthetic performance of four week old plants and flowering plants indicate that the impact of the *au* mutation on growth and development becomes reduced with increasing age of the mutant plants (Becker et al., 1992).

The survival of *au* mutant in normal daylight and restoration of RL-impaired responses to WT levels under WL such as fresh mass accumulation, chlorophyll accumulation, photosynthetic oxygen evolution, and NiR photoinduction, suggest that a simultaneous operation of a BL receptor and residual phytochrome may be restoring the above responses under WL and may be helping the mutant to complete its life cycle. Such a type of interdependent co-action (Mohr

1986) between phytochrome and specific BL/UVA receptor was shown in the control of stem extension growth responses to BL in *au* mutant (Casal 1994). It was also suggested that *au* mutant is deficient in a pool of phytochrome apparently not essential for the interdependent co-action between phytochrome and BL/UVA receptor observed for stem extension growth (Casal 1994). Furthermore, the observation of a BL-promoted *in vitro* bud regeneration both in the presence of high and low IAA concentration in *au* mutant (Mercenaro et al., 1994), indicates the presence of functional BL receptor in it.

The *hp* mutant of tomato is characterized by the presence of phytochrome content of etiolated seedlings (predominantly phytochrome A) and the physiological characteristics of phytochrome system similar to that in WT (Adamse et al., 1989; Peters et al., 1989). At the same time it exhibits exaggerated phytochrome responses such as maximum anthocyanin synthesis, and hypocotyl growth inhibition in RL alone, and does not require co-action of the BL photoreceptor and phytochrome for normal development. The results in the present thesis indicate that *hp* mutant is quite opposite to *au* mutant with respect to photoresponses which were deficient in *au* such as high dark levels of all enzyme activities assayed, higher magnitude induction of all the enzymes studied high amount of chlorophyll, high amount of fresh mass accumulation longer, and higher density of hairs on hypocotyl, suggesting that the phytochrome species whose responsiveness is amplified in the *hp* mutant and the phytochrome species which is deficient in *au* may both be same. Furthermore, the observation of normal phytochrome responses such as hypocotyl elongation growth in response to EODFR treatment and low

R:FR ratio in both *au* and *hp* mutants will further strengthen the above suggestion. Therefore, the molecular analysis of *hp* mutation may result in identification of a signaling component specific to a phytochrome species that is deficient in *au*, which is predominantly phytochrome A.

6. SUMMARY

Light plays a paramount role in directing growth and differentiation in plants. Higher plants sense their ambient light environment using three photoreceptors which independently or cooperatively monitor the light impinging on plants. Among these, the molecular identity of the UV-B photoreceptor is unknown; for the blue/UV-A photoreceptor, a gene encoding a putative photoreceptor candidate has been cloned. In contrast the phytochrome detecting the red region of the spectrum has been purified, and the genes encoding for it have been cloned. It has also been clearly shown that phytochrome regulated photoresponses ensue from photoregulation of gene expression leading to photomorphogenesis. In addition it is now apparent that phytochrome consists of multiple species, encoded by a small multigene family. Therefore, different species of phytochrome may have distinct functions that perhaps induce discrete signal chains.

The signal transduction pathway triggered after activation of photoreceptors is currently being intensively investigated by a variety of techniques such as production of transgenics, biochemical estimations of levels and activation of putative components, and usage of photomorphogenic mutants. Among these techniques, mutants are the most convenient material to decipher the molecular action of photoreceptors and components participating in signal transmission. Analysis of these mutants has considerably advanced our knowledge of

molecular events associated with photomorphogenesis. In the present study we have used a photomorphogenic mutants of tomato, namely, *au* mutant, which is deficient in spectrally active phytochrome A in young seedlings but possesses spectrally active phytochrome B in green plants; *hp* mutant, which shows exaggerated response to light, and double mutant of *au* and *hp*, to learn about molecular events associated with photomorphogenesis of tomato.

Deficiency of phytochrome in *au* altered several facets of seedling development, which were clearly apparent at the morphological level in light-grown seedlings. In general, WT, *au*, and *hp* seedlings were morphologically similar in darkness; however, while a transfer to light initiated a rapid photomorphogenesis in WT, and *hp*, *au* was very slow to respond. This was clearly demonstrated by the lack of several light-induced responses in *au* mutant such as inhibition of hypocotyl elongation, cotyledon expansion, initiation and elongation of hair on hypocotyls and pigment accumulation. In contrast, in *hp* the above responses were many times higher than in WT, while *au,hp* mutant responded like *au.*

Dark-grown *au* seedlings possessed very little hair on hypocotyls as compared to WT and *hp.* Even in seedlings grown under RL, hair was shorter and less in *au* as compared to WT and *hp* grown under RL. Similarly, *au* seedlings also lacked light-induced fresh mass and chlorophyll accumulation in continuous RL. However, in WL-grown seedlings the magnitude of cotyledon expansion and chlorophyll accumulation was restored nearly to WT levels. In contrast, in *hp*

seedlings,, chlorophyll accumulation was significantly higher than in WT, and fresh mass accumulation was equal to WT.

Tomato WT seedlings grown under light characteristically accumulate anthocyanin in several regions of seedlings, particularly in the hypocotyl hook and region close to root. It is more strikingly visible in *hp* seedlings than WT with a greater amount of anthocyanin in seedlings. However *au* and *au,hp* seedlings completely lack this light-mediated anthocyanin induction. In most systems, the above photoinduction of anthocyanin is preceded by an enhancement in the PAL level. PAL catalyzes the deamination of phenylalanine, which is a key precursor in the complex anthocyanin biosynthetic pathway. In view of this causal link between anthocyanin accumulation and regulation of PAL, the interrelationship between photoregulation of PAL and photoinduction of anthocyanin accumulation was studied in tomato WT and its photomorphogenic mutants.

Irrespective of the deficiency of phytochrome in *au* and *au,hp* seedlings, photoinduction of PAL was observed in cotyledons and hypocotyl of these mutants. The profile of PAL photoinduction in *au* and *au,hp* was essentially similar to WT and *hp*, except that the magnitude of photoinduction was lower in *au.* On the contrary, in hypocotyls of *hp* seedlings, photoinduction of PAL was about 3 fold higher than that in WT. Western blot analysis of PAL by using polyclonal antisera against tobacco PAL, confirmed that the RL-mediated PAL increase results from accumulation of PAL protein in *au, hp,* and WT seedlings. Comparison of profiles of anthocyanin biosynthesis and PAL photoinduction revealed

that phytochrome-mediated induction of PAL and anthocyanin in the tomato seedlings are however not correlated. A lack of correlation between PAL induction and anthocyanin accumulation was clearly apparent by the fact that though *au* and *au,hp* seedlings show a photoinduction of PAL level like WT, they do not form anthocyanin.

Earlier studies on photoinduction of several nuclear-encoded plastidic genes revealed that *au* seedlings lack photoinduction of plastidic proteins and need blue light to elicit Pfr action on these proteins. Our study indicates that even a brief pulse of RL can induce PAL activity in *au* seedlings. Since PAL is a cytosolic enzyme, it was investigated whether other cytosolic enzymes are similarly induced in *au*. Photoinduction of cytosolic enzymes NR and amylase was compared to a plastidic enzyme NiR in *au* mutant, and to WT and *hp*.

In the wild type and *hp*, both brief RL pulses and continuous RL induced amylase, NR, and NiR activities. In *au*, brief pulses of RL were ineffective to induce these enzymes and continuous RL induced only amylase and NR activities. However, RL-mediated NR and amylase-induction profiles in *au* were similar to those in WT and *hp*, except that the magnitude of enzyme induction in *au* was considerably lower than in WT and *hp*. By contrast, the NiR induction was absent in *au* mutant.

A blue light (BL) pretreatment prior to continuous RL exposure was ineffective in inducing NiR activity in *au*. Only continuous white light could elicit a photoinduction of NiR in it. Since RL induces NiR transcript accumulation in *au* (Becker et al., 1992) it was considered

possible that lack of NiR induction may result from a defective plastid development. Probably a simultaneous operation of BL photoreceptor and residual phytochrome under continuous WL restores the photoinduction of NiR by stimulating Chloroplast development in *au* seedlings.

The likelihood of a delayed plastid development or abnormal plastid development in *au* mutant was investigated by studying the photoinduction of Chloroplast differentiation which was monitored by its capacity to acquire *in vivo* light-mediated carbon dioxide dependent oxygen evolution. Monitoring of carbon dioxide dependent oxygen evolution in intact seedlings under continuous RL and WL after 96 hours in the dark, revealed that the amount of oxygen evolved in tomato seedlings is directly proportional to the amount of chlorophyll accumulated. In *au*, the capacity to evolve oxygen is restored slowly to near normal levels along with the restoration of chlorophyll accumulation under WL, which were earlier impaired under RL. In contrast, in *hp* cotyledons, although there was more chlorophyll accumulation than in WT, the efficiency of these seedlings to fix carbon dioxide did not increase accordingly.

In summary, the results of the present study show that the *au* mutant of tomato, though severely depleted in spectrally active phytochrome, retains at a reduced level several of the Pfr-induced responses. In contrast, the *hp* mutant exhibits a very high magnitude of all the responses studied so far. Though the *au* mutant lacks several RL-mediated responses such as inhibition of hypocotyl elongation,

induction and elongation of hair on hypccotyl, cotyledon expansion, accumulation of chlorophyll and anthocyanin, which probably stems from deficiency of phytochrome A, it still possesses photoinduction of PAL. It may well be possible that anthocyanin accumulation is under the control of phytochrome A, and PAL induction is mediated by the residual phytochrome. Similarly the retention of photoinduction of other cytosolic enzymes may be mediated by residual phytochrome pool. Absence of the NiR photoinduction in *au* may be either due to phytochrome A deficiency or defective Chloroplast development. Since the *au* mutant possesses a delayed and sluggish acquisition of photosynthetic oxygen evolution, it indicates that in addition to phytochrome deficiency, *au* also has a block in Chloroplast development.

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Photoregulation of Phenylalanine Ammonia Lyase is not Correlated with Anthocyanin Induction in Photomorphogenetic Mutants of Tomato *(Lycopersicon esculentum)*

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Photoregulation of phenylalanine ammonia lyase (PAL)(EC 4.3.1.5) was analyzed in wild type (WT) and mutants: phytochrome deficient-*aurea* (aw), high pigment exhibiting exaggerated phytochrome response *(hp)* and the double mutant (*au,hp*) of tomato (*Lycopersicon esculentum* (Mill.) cv. Ailsa Craig). Red light, acting via phytochrome, stimulates PAL activity in cotyledons and hypocotyls of tomato seedlings. The time course of photoinduction of PAL in cotyledons of the mutants (*au* and *au.hp)* and WT seedlings has a peak of activity at 4 h, after which the activity falls sharply, except in *hp* seedlings where activity is maintained at a high level. In hypocotyls, photoinduction of PAL also shows an initial rise, reaching a maximum at 3 h, followed by a sharp decline in the mutants *(au* and *au,hp)* and WT seedlings. However in *hp* seedlings photoinduction of PAL is about 3 fold that in WT. The photoinduction of PAL appears to be dependent on de novo synthesis of protein and nucleic acids. The use of a PAL specific inhibitor α-aminooxy β-phenylpropionic acid indicated that PAL is an essential component of the anthocyanin biosynthetic pathway in the tomato seedlings. However, a comparison of anthocyanin biosynthesis [Adamse et al. (1989) *Photochem. Photobiol.* 50: 107] and PAL photoinduction data revealed that phytochrome mediated induction of PAL and anthocyanin in the tomato seedlings are not correlated. While *au* and *au,hp* mutant seedlings show a similar increase in PAL level as in the WT, there is little formation of anthocyanin in these mutant seedlings. The results indicate that, in contrast to the photoregulation of anthocyanin synthesis which is dependent on the presence of the labile phytochrome (*l*P) pool in tomato seedlings, the photoinduction of PAL is mediated via a small pool of phytochrome in *au* mutant: stable phytochrome (*s*P) or a residual *l*P pool.

Key words: Anthocyanin — *Lycopersicon esculentum* — Phenylalanine ammonia lyase — Photomorphogenesis — Phytochrome — Tomato.

The growth and development of higher plants is influenced by the light environment, in which the photoreceptor phytochrome plays an important role. Despite significant progress made during the past decade in understanding the molecular properties of phytochrome (Furuya 1989) and its influence on gene expression of a few abundant proteins (Jenkins 1991), the molecular mechanism of the signal transduction chain originating after conversion of phytochrome to physiologically active P_{FR} form is not yet fully understood. Moreover, phytochrome is present in multiple molecular types in vivo (Furuya 1989, Abe et al. 1989, Sharrock and Quail 1989) and these different types may independently control particular photoresponses (Furuya et al. 1991, Ehmann et al. 1991).

The discovery of various photomorphogenetic mutants in tomato, *Arabidopsis* and cucumber, which have either photoreceptor deficiency or a modified signal trans-

Abbreviations: *au, aurea*; D, darkness; FR, far-red light; *hp*, high pigment; *l*P, lable phytochrome; PAL, phenylalanine ammonia lyase; RL, red light; *s*P, stable phytochrome; WT, wild type.

duction chain, has provided new tools to investigate and localize the steps involved between the perception of light by phytochrome and final expression of the photoresponse (Adamse et al. 1988b, Chory et al. 1989). In tomato, *au* mutants are deficient in the photoreceptor phytochrome in etiolated seedlings (Parks et al. 1987, Sharrock et al. 1988) and show reduced phytochrome mediated photoresponses particularly during de-etiolation (Adamse et al. 1988a). In contrast, in *hp* mutants of tomato, although the concentration of phytochrome is the same as that in the WT, the regulation of several photoresponses is greatly enhanced in magnitude, by an apparent modification in the signal transduction chain (Peters et al. 1989).

In higher plants, photoregulation of biosynthesis of anthocyanin in developing seedlings is a useful model system to study the interrelationship between photoreceptor activation and accumulation of the end product (Mancinelli 1985). In tomato, photoregulation of anthocyanin synthesis in various photomorphogenetic mutants has been employed to investigate the mode of primary action of phytochrome in bringing about this photoresponse (Adamse 1988). These studies revealed that the photocontrol of anthocyanin is dependent on the availability of the bulk labile phytochrome pool. In *au*-mutant seedlings the photoinduction of anthocyanin is nearly abolished, due to the phytochrome deficiency (Adamse et al. 1988b,1989). In contrast, in *hp* seedlings despite having the same level of phytochrome as in WT, the response to light is amplified 8-10 fold (Adamse et al. 1989, Peters et al. 1989).

The anthocyanin biosynthetic pathway in plants takes place via a complex pathway, one precursor is phenylalanine, which is first deaminated by the enzyme PAL. and after a series of reactions the end product of which (4-coumaroyl-Co A) is catalyzed by chalcone synthase to combine with 3 molecules of malonyl-Co A and is a key nodal point for the biosynthesis of anthocyanin. Both these enzymes show photoregulation of the expression of their respective genes (Beggs et al. 1986, Hahlbrock and Scheel 1989, Ehmann et al. 1991). In several systems the photoinduction of anthocyanin biosynthesis is preceded by an enhancement in the PAL level in the photoresponsive tissue (Beggs et al. 1986, 1987). In view of this causal interrelationship between anthocyanin accumulation and regulation of PAL we have conducted a study on photoregulation of PAL in seedlings of different photomorphogenetic mutants of tomato.

Materials and Methods

Growth of seedlings—Seeds of tomato (*Lycopersicon esculentum* Mill. cv. "Ailsa Craig") WT, *hp, au* and *au,hp* were used in this study. Seeds were sown in transparent plastic boxes (9 cm / × 9 cm *b* x 4.5 cm *h),* each containing 36 seeds, on 20 ml of 0.5% agar prepared with 1/10 concentration of MS medium (inorganic salts only, Murashige and Skoog 1962) at 25°C. The seedlings were grown in absolute darkness till the time point of transfer to continuous RL or RL and FR pulse treatments. The light sources for RL (0.6 W m^{-2}) and FR (3.5 W m^{-2}) were similar to those described in Manga and Sharma (1985).

PAL extraction and assay—Twenty hypocotyls or pairs of cotyledons were homogenized at 4°C in a pestle and mortar with 0.2 g of sea sand and 0.15 g of polyvinylpolypyrrolidone in 3 ml 0.1 M borate buffer (pH 8.8) containing 54 mM mercaptoethanol. The homogenate was centrifuged at 18,200 × *g* for 30 min at 4°C and supernatant was applied to a Sephadex G-25 column (size: 2 cm diameter x 10 cm) equilibriated with 0.1 M borate buffer (pH 8.8). The fractions constituting void volume were pooled together and were used for PAL assay. The PAL assay was performed at 25°C in an assay mixture consisting of 2 ml of enzyme extract and 1 ml of 50 mM L-phenylalanine (Saunders and McClure 1975). The PAL activity was assayed by monitoring the increase in the absorbance at 290 nm against a control without phenylalanine over a period of 4 h at 1 h intervals, in a Hitachi 557 spectrophotometer. The rate of appearance of *trans*-cinnamic acid was taken as a measure of enzyme activity using increase in 0.01*A* at 290 nm as 3.09 nmoles of *trans*-cinnamic acid formed (Saunders and McClure 1975). The PAL activity is expressed in pkat (pmol *trans*-cinnamic acid formed per second) per pair of cotyledons or per hypocotyl.

Anthocyanin assay—Ten seedlings of uniform height were taken and cotyledons and hypocotyls were extracted separately with 1.2 ml of acidified methanol (1% HC1, w/v) for 48 h in darkness with constant shaking. A Folch partitioning was performed after adding 0.9 ml of water and 2.4 ml of chloroform to the extracts and centrifugation for 20 min at 1,600 × *g*. The absorbance of the top phase was determined and values are expressed as $A_{535nm}/10$ hypocotyls or pairs of cotyledons (Adamse 1988).

Inhibitors—*a*-Aminooxy*β*-phenylpropionic acid (0.4 mM), cycloheximide (25 μg ml^{-1}), cordycepin (200 μg ml^{-1}), puromycin (200 μg ml^{-1}) and actinomycin-D (100 μg ml^{-1}) were used in different experiments. The inhibitors (3 ml, each) were sprayed on the seedlings under a green safe light (> 1 μW m^{-2}) and seedlings were immediately returned to darkness for the next 2 h before transferring them into RL.

Results

The influence of phytochrome on the PAL activity in tomato seedlings was estimated by growing mutant *(au; hp* and *au,hp)* and WT seedlings in continuous RL and in the darkness from the sowing. Figure 1 shows that RL stimulated the PAL level in the cotyledons of the tomato mutant and WT seedlings. The magnitude of photoinduction was

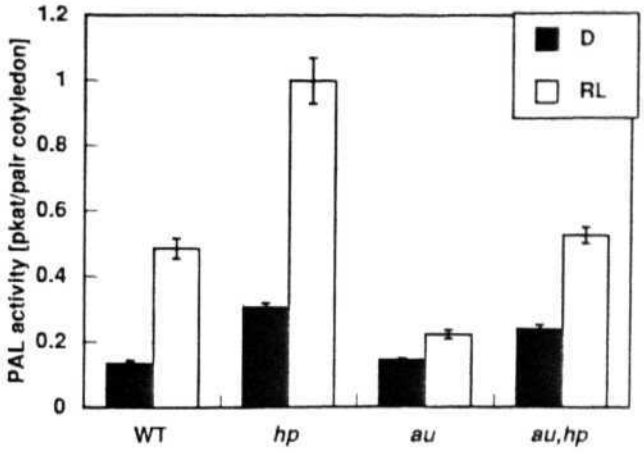

Fig. 1 Phenylalanine ammonia lyase (PAL) activity in cotyledons of tomato seedlings. The PAl. activity was measured at 96 h in WT and mutant seedlings grown in continuous RL and complete darkness (D) from sowing.

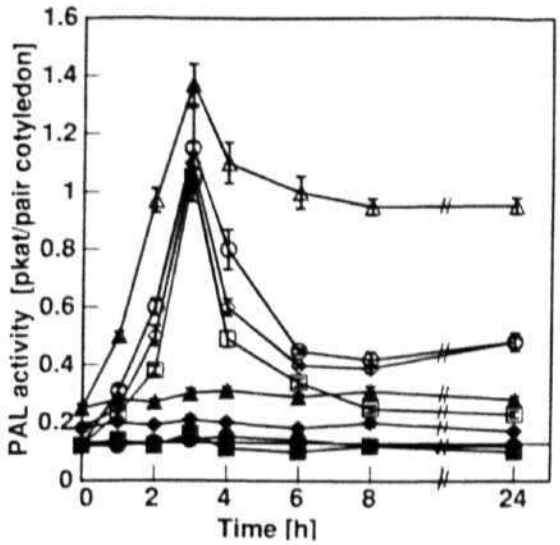

Fig. 2 Time course of PAL activity in cotyledons of tomato seedlings. Seedlings were grown in darkness up to % h from sowing and then were transferred to continuous RL (open symbols). The control seedlings were maintained in continuous darkness (closed symbols). WT (, •), *hp* (, A), *au* (. •), *au.hp* (◇, •).

greatest in *hp*, decreased in the WT and *au,hp* and was least in *au* seedlings. In seedlings transferred to the continuous RL after 72 h in darkness the level of PAL in the different genotypes followed a similar pattern to Fig. 1 (data not shown).

The activity of PAL was also estimated in the cotyledons and the hypocotyl of tomato seedlings. Figure 2 shows the time course of PAL activity in the tomato cotyledons. In all the mutants and WT seedlings, transfer from darkness to continuous RL resulted in a rapid enhancement in PAL activity, attaining a peak 3 h after the onset of the RL. Thereafter, PAL activity gradually declined, nevertheless it was maintained at a significantly higher level than the dark controls after 24 h RL. In the case of *hp* the PAL activity after attaining a peak level declined only slightly compared to other genotypes.

The time course of photoinduction of PAL level in the hypocotyl followed a different pattern than that in the cotyledons (Fig. 3). In the *hp* mutants PAL level increased at a more rapid rate than the other mutants and WT seedlings and attained a 5-fold higher level 4 h after the onset of RL irradiation. Thereafter, the level of PAL gradually declined to the dark control levels 8 h after the onset of RL for all the genotypes. In comparison to the *hp* mutant, the magnitude of PAL induction in WT, *au* and *au,hp* seedlings was much less, but even in these seedlings the photoinduction of PAL lasted only for a period of 8 h with a peak level being attained 4 h after the onset of RL. In comparison to cotyledons, photoinduction of PAL activity in hypocotyls was delayed by about 1 h.

Table 1 The effect of brief red and far-red irradiation on PAL activity of tomato cotyledons

Irradiation program	PAL activity ± SE (pkat (pair cotyledon)$^{-1}$)			
	WT	*hp*	*au*	*au,hp*
Control (96 h D)	0.12±0.009	0.25±0.014	0.12±0.009	0.18±0.011
+ 3 h D	0.14±0.007	0.30±0.016	0.13±0.002	0.21±0.014
+ 3 h RL	1.20±0.140	1.37±0.070	1.09±0.084	1.10±0.070
+ 5 min RL + 3 h D	1.05±0.127	1.15±0.070	0.91±0.084	0.95±0.127
+ 5 min FR + 3 h D	0.20±0.011	0.51±0.014	0.19±0.005	0.39±0.014
+ 5 min RL + 5 min FR + 3 h D	0.17±0.005	0.42±0.028	0.16±0.005	0.32±0.014

Seedlings were grown in darkness (D) from sowing. At 96 h from sowing, they were subjected to RL and FR irradiations as indicated above.

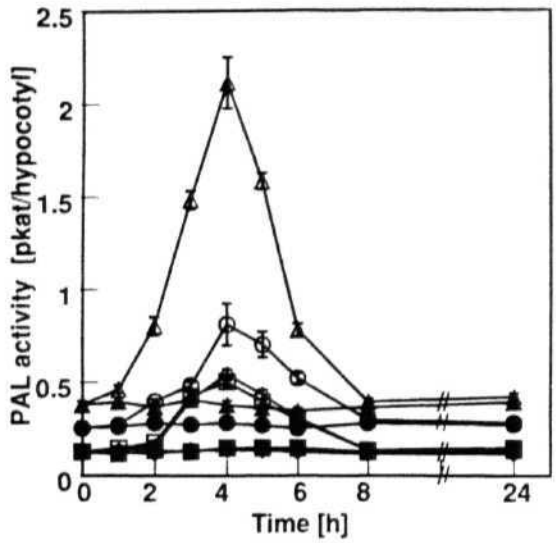

Fig. 3 Time course of PA1 activity in hypocotyls of tomato seedlings. Seedlings were grown in darkness up to 96 h from sowing and then were transferred to continuous RL (open symbols). **The** control seedlings were maintained in continuous darkness (closed symbols). WT (, •), *hp* (, A), *au* (, •), *au.hp* (◇, •).

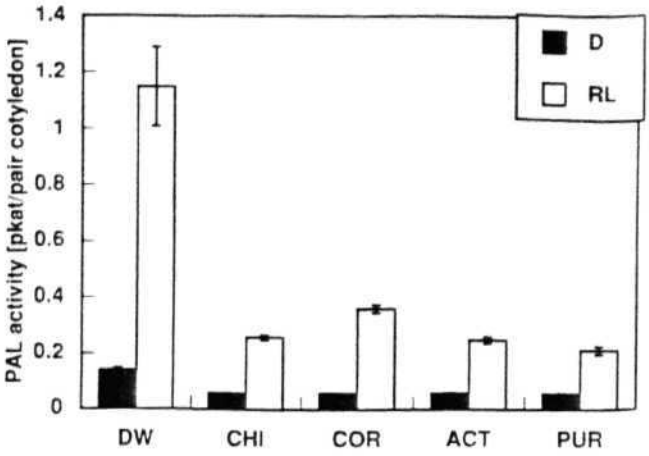

Fig. 4 Effect of inhibitors of RNA (actinomycin-D, cordycepin) and protein (cycloheximide, puromycin) synthesis on photoinduction of PAL activity. At 96 h from sowing, dark-grown WT tomato seedlings were transferred to continuous RL. The PAL activity was measured in the cotyledons after 3 h of continuous RL irradiation. The seedlings were treated with above inhibitors 2 h before transferring to RL. The control seedlings in darkness (D) were similarly treated with distilled water or inhibitors. DW, distilled water; CHI, cycloheximide (25 μg ml^{-1}); COR, cordycepin (200 μg ml^{-1}); ACT, actinomycin-D (100 μg ml^{-1}); PUR. puromycin (200 μg ml^{-1}).

The participation of phytochrome in the photoinduction of PAL was confirmed by the R/FR reversibility experiments. Tables 1 and 2 show that in tomato seedlings RL acting via phytochrome significantly increases PAL activity in both cotyledons and the hypocotyl. A single pulse of RL is nearly as effective as continuous RL, and if it is followed by a FR pulse the effect of the RL pulse on PAL induction is significantly negated. Far red light alone also induced about a 1.5-2-fold increase in the PAL level in both the cotyledons and in the hypocotyl. In the cotyledons there was little difference in the magnitude of RL-mediated induction of PAL level between mutants and WT seedlings. In contrast, in the hypocotyl of the *hp* mutant the RL-mediated induction of PAL level was much greater in magnitude than that of the WT. However, RL-mediated induction is lower in magnitude in the *au* and the *au,hp* mutant hypocotyls. Interestingly in the *hp* mutant, both in the hypocotyl and in the cotyledons, the level of PAL in seedlings grown in absolute darkness was 1.5-2-fold higher than the corresponding WT seedlings, while in dark-grown *au* seedlings the PAL level was equivalent or lower than WT in the cotyledons and in the hypocotyl, respectively.

The dependence of photoinduction of PAL on nucleic

Table 2 The effect of brief red and far-red irradiation on PAL activity of tomato hypocotyls

Irradiation program	PAL activity ± SE (pkat hypocotyl^{-1})			
	WT	*hp*	*au*	*au,hp*
Control(96 h D)	0.25±0.014	0.37±0.021	0.12±0.009	0.11±0.001
+ 4 h D	0.27±0.012	0.37±0.029	0.11±0.007	0.13±0.002
+ 4 h RL	0.81±0.113	2.11±0.140	0.53±0.025	0.54±0.028
+ 5 min RL + 4 h D	0.71±0.070	1.75±0.049	0.50±0.025	0.51±0.025
+ 5 min FR + 4 h D	0.38±0.014	0.62±0.070	0.19±0.011	0.21±0.014
+ 5 min RL + 5 min FR + 4 h D	0.30±0.015	0.51±0.029	0.14±0.007	0.19±0.014

Seedlings were grown in darkness (D) from sowing. At 96 h from sowing, they were subjected to RL and FR irradiations as indicated above.

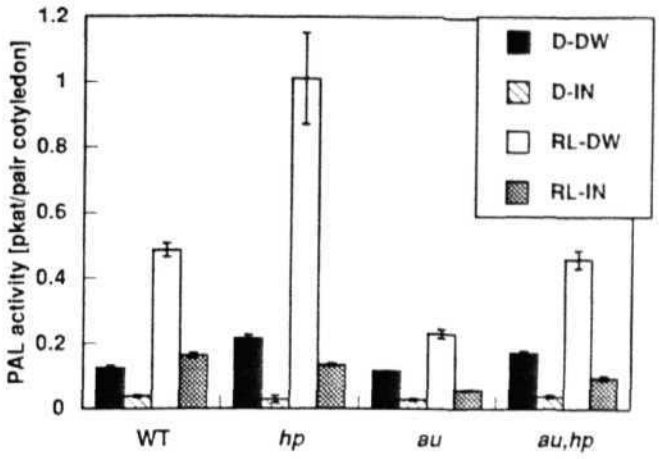

Fig. 5 Effect of a-aminooxy β-phenylpropionic acid on photoinduction of PAL activity. Tomato seedlings were grown in darkness (D). At 72 h from sowing, a set of seedlings was transferred to continuous RL. The PAL activity was measured in the cotyledons after 24 h of continuous RL. The seedlings were treated with the above inhibitor (0.4 mM) (IN) 2 h before transferring to RL. The control seedlings were similarly treated with distilled water (DW).

acid (RNA) and protein synthesis in tomato seedlings was investigated by using inhibitors of these processes. The application of these inhibitors to seedlings prior to the onset of RL irradiation significantly inhibited the photoinduction of PAL in the cotyledons (Fig. 4) and the hypocotyls of WT and the mutant seedlings (data not shown).

Transverse sections of cotyledons and the hypocotyl revealed that in tomato seedlings anthocyanin is localized in the epidermal layer of hypocotyl and lower epidermis of cotyledons. The role of the PAL in mediating anthocyanin biosynthesis was investigated by using a PAL specific inhibitor a-aminooxy β-phenylpropionic acid (Amrhein and Godeke 1977) which inhibits the PAL by binding to its active site. Figure 5 shows that application of this inhibitor brings about a reduction of the PAL activity in darkness and abolishes the photoinduced increase in PAL activity in WT and the mutants seedlings. The estimation of anthocyanin level in cotyledons and the hypocotyl of tomato seedlings treated with a-aminooxy β-phenylpropionic acid, revealed that the inhibitor treatment to a large extent also abolished RL-mediated induction of anthocyanin in *hp* and WT seedlings (Table 3).

Discussion

Photomorphogenetic mutants provide excellent tools to investigate the photoreceptors involved in perception of light signals and the subsequent elements of the signal transduction chain leading to the terminal response (Adamse 1988). In the present study we have employed tomato mutants to investigate the steps involved in the photoregulation of anthocyanin biosynthesis in these plants. In many plants species the photostimulation of anthocyanin accumulation is preceded by an enhancement of PAL activity, a key enzyme involved in the regulation of phenylpropanoid biosynthesis in plants (Beggs et al. 1987, Brödenfeldt and Mohr 1988, Hahlbrock and Scheel 1989). In tomato, RL initiates a strong photoinduction in PAL activity in both the hypocotyl and the cotyledons (Figs. 1-3). This photoinduction of PAL in tomato can be attributed to phytochrome, since the increase in PAL activity by a brief RL pulse is reversed by a subsequent pulse of FR (Lercari et al. 1982). Interestingly, this photoresponse is observed in all the tomato mutant seedlings, even though the *au* and *au.hp* mutants possess no spectrophotometrically detectable

Table 3 The anthocyanin content of tomato seedlings grown in the presence (+) or absence of (−) of α-aminooxy β-phenylpropionic acid

	Anthocyanin content ($A_{535\,nm} \times 10^{-3}$)/10 Organs							
	Cotyledons				Hypocotyls			
	96 h D		72 h D + 24 h RL		96 h D		72 h D + 24 h RL	
	−	+	−	+	−	+	−	+
WT	2	1	8	2	3	1	46	16
hp	3	1	69	12	4	1	555	155
au	0	0	0	0	0	0	2	1
au,hp	0	0	0	0	1	0	3	1

Seedlings were grown in the darkness (D). At 72 h from sowing, a set of seedlings was transferred to continuous RL. The anthocyanin content was measured, in hypocotyls and cotyledons separately, after 24 h continuous RL. The seedlings were treated with a-aminooxy β-phenylpropionic acid 2 h before transferring to RL. The control seedlings were similarly treated with distilled water. The standard error for the anthocyanin values presented in the table was < 5%.

phytochrome in dark-grown seedlings (Parks et al. 1987, Adamse 1988) and are almost 'red blind', not completely de-etiolating under RL. The close similarity in the time courses of photoinduction of PAL activity in both *au* and *au.hp* double mutant to WT indicates that the deficiency in bulk phytochrome does not impair the photoregulation of PAL in these mutants. The sustainment of PAL activity at a higher level in the cotyledon and greater magnitude of photoinduction of PAL in the hypocotyl in *hp* mutants are in accordance with it being a signal transduction amplification mutant (Adamse et al. 1988b, Peters el al. 1989). The sustainment of a high level of PAL in cotyledons of *hp* mutants may result from either a reduced rate of PAL degradation or a sustained synthesis of PAL at a steady level. In the *hp* mutant there is a general increase in the PAL level since even in total darkness it is twice that in the WT seedlings.

In tomato seedlings of the *au* genotype the R/FR reversible photoresponses are lacking or severely reduced at the stage of de-etiolation, presumably due to significant reduction in the level of labile phytochrome pool (Adamse et al. 1988b). In the present study it is evident that the FR reversible response on the PAL level in both the hypocotyl and in the cotyledons of *au* and *au,hp* mutant is nearly equivalent to that in WT seedlings. In previous studies with *au* seedlings such a reversion of an inductive RL pulse by FR has not been observed, except for the photoregulation of *Cab* gene expression (Sharrock et al. 1988, Oelmüller and Kendrick 1991), which is however greatly reduced in magnitude compared to the WT. Since *au* is deficient in *lP* it is possible that the above PAL response is mediated by the *sP* which has been shown to accumulate in light-grown *au* plants (Adamse et al. 1988a) and which regulates photoresponses in a similar fashion to light-grown WT plants (L6pez-Juez et al. 1990). The R/FR reversible photoregulation of PAL observed in WT tomato and the mutant seedlings studied here could be under the control of the *sP* pool. Alternatively a low residual *lP* pool below detection limits can not be excluded.

The photoinduction of PAL in tomato seedlings does not show good correlation with stimulation of anthocyanin biosynthesis in the same seedlings (Adamse et al. 1989). In the *au* and *au.hp* mutant seedlings little anthocyanin is produced, but the time courses of increase in PAL level in respect to RL is similar to WT. Similarly in the hypocotyl a correlation between PAL induction and anthocyanin level is not evident. In tomato seedlings, the majority of anthocyanin biosynthesis takes place in the hypocotyl, but P_{FR}-mediated induction of PAL in the hypocotyl and its decrease is completed within 8 h, before the onset of the sustained increase in the anthocyanin level (Peters et al. 1991). Although RL irradiation leads to a greater enhancement in the level of PAL in the hypocotyl of *hp* mutants, PAL activity is also significantly enhanced in WT, *au* and *au.hp* mutant seedlings, which fail to produce much anthocyanin (Table 3). Furthermore, in *au* seedlings a single pulse of RL significantly increases PAL level, but the same *au* seedlings do not show any detectable induction of anthocyanin with a single RL pulse (Adamse et al. 1989). Moreover, in all the genotypes used except the *hp* mutant, a blue light pretreatment is required to observe the phytochrome regulation of anthocyanin induction (Adamse 1988, Mancinelli 1985), while in the present study the genotypes used exhibited R/FR reversible photoregulation of PAL without a blue light pretreatment.

It is evident from the foregoing discussion that there is no strict correlation between the photoinduction of PAL measured here and the biosynthesis of anthocyanin in tomato seedlings. The lack of correlation between photoinduction of anthocyanin biosynthesis and PAL has been noticed in other systems on the basis of lack of coordination in the kinetics of photoinduction of PAL and anthocyanin accumulation (Brodenfeldt and Mohr 1988). While our studies rule out a direct correlation between a phytochrome mediated induction of the major pool of PAL and anthocyanin biosynthesis, they do not rule out a key role played by PAL in anthocyanin biosynthetic pathway. Since the inhibition of PAL activity in vivo by α-aminooxy β-phenylpropionic acid, strongly reduces the photoinduction of anthocyanin in tomato seedlings (Table 3); PAL activity is a prerequisite for anthocyanin synthesis. In mustard seedlings where anthocyanin is localized in the lower epidermis of cotyledons, Beggs et al. (1987) after dissecting cotyledons into lower and upper parts, found a correlation between PAL and anthocyanin increase in the lower epidermis during first few hours of anthocyanin accumulation. Since anthocyanin in tomato seedlings is also strictly localized in the lower epidermal layer of the cotyledons and the sub-epidermal layer of the hypocotyl, it is possible that that PAL activity in these epidermal layers follows a different kinetic pattern of photoregulation than the total PAL pool in the whole organs. The inhibition of anthocyanin accumulation by a-aminooxy β-phenylpropionic acid (Table 3) indicates that a minor pool of PAL may play a role in anthocyanin accumulation. Although such tissue specific correlation between photoregulation of PAL and end product accumulation has not been firmly established for PAL, another enzyme chalcone synthase which plays an important role in regulating anthocyanin synthesis, clearly shows a tissue specific distribution and induction by light (Ehmann et al. 1991, Schmelzer et al. 1988). The inhibition of PAL photoinduction by RNA and protein synthesis inhibitors (Fig. 4) indicates that PAL is de novo synthesized in tomato seedlings (Brodenfeldt and Mohr 1986).

In summary, it is evident that although phytochrome mediates PAL induction, the major pool of PAL present in tomato seedlings does not participate in controlling the an-

thocyanin level upon exposure of etiolated seedlings to continuous RL. The results presented in this study also indicate that, while the photoregulation of anthocyanin synthesis is dependent on photoperception by fP, the photoregulation of PAL is proposed to be mediated by the low sP pool in tomato seedlings.

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Retention of Photoinduction of Cytosolic Enzymes in *aurea* Mutant of Tomato (*Lycopersicon esculentum*)[1]

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The tomato *(Lycopersicon esculentum* Mill.) *aurea (au)* mutant has been characterized as a phytochrome-deficient mutant lacking spectrally detectable phytochrome A in etiolated **seedlings**. Seedlings of *au* grown under red light (**RL**) lack phytochrome regulation of nuclear genes encoding plastidic proteins, possess ill-developed chloroplasts, and are slow to de-etiolate. In the present study, **the** effect of phytochrome deficiency on photoinduction of **enzymes** in etiolated *au* seedlings was investigated. The photoinduction of the cytosolic enzymes amylase and nitrate reductase (**NR**) and of the plastidic enzyme nitrite reductase (NiR) in *au* was compared with that in the isogenic wild-type (WT) tomato and the *high-pigment (hp)* mutant with exaggerated phytochrome response. **In** WT and *hp,* both brief RL pulses and continuous RL induced amylase, NR, **and** NiR activities, whereas in *au* no photoinduction of enzymes was observed with brief RL pulses, and continuous RL induced only amylase and NR activities. The time courses of photoinduction of NR and amylase in *au* under continuous RL followed patterns qualitatively similar to *hp* and WT. A blue-light pretreatment prior to continuous RL exposure was ineffective in inducing NiR activity in au. Only continuous white light could elicit a **photoinduction** of NiR in au seedlings. The norflurazon-triggered loss of photoinduction of NiR in WT and *hp* indicated that NiR photoinduction depended on Chloroplast biogenesis. The results indicate that observed photoinduction of NR and amylase in *au* may be mediated by a residual phytochrome pool.

In higher plants the light environment is sensed by three photoreceptors: L'V-B, blue/UV-A, and phytochrome. Of these, phytochrome is the only photoreceptor whose molecular identity is known (Quail, 1991). Phytochrome plays a prominent role in detecting light environment, alone or in conjunction with other photoreceptors, throughout the life history of plants. The molecular analysis of phytochrome genes in *Arabidopsis thaliana* has indicated that a small multigene family encodes phytochromes, of which at least five phytochrome genes, *PHYA* through *PHYE,* have been identified (Sharrock and Quail, 1989; Quail, 1991). The investigations on expression of *PHY* genes have established that the apoprotein of the photolabile phytochrome present in etiolated seedlings is encoded by the *PHYA* gene, whereas *PHYB* and *PHYC* encode the apoproteins of photostable phytochromes (Quail. 1991; Furuya, 1993) The presence of multiple phytochrome species has led to the suggestion that different phytochrome species may perform discrete functions via distinct signal-transduction pathways (Smith and Whitelam. 1990) Considering that in a light-grown plant all phytochrome species may function concurrently, mutants lacking one or more phytochrome species are valuable to distinguish the role played by different phytochrome species in regulating plant development (Reed ft al., 1992)

Phytochrome-deficient mutants have been reported in *Arabidopsis,* tomato *(Lycopersicon esculentum),* sorghum, *Brassica,* and cucumber (Kendrick and Nagatani, 1991; Reed et al., 1992), and the relative functions of species of phytochrome in plant development have been inferred from observations of the physiological responses and studies on the photoregulation of gene expression in these mutants (Smith and Whitelam, 1990) Studies of *PHYB*-deficient mutants reveal that phytochrome B plays a major role in inhibition of hypocotyl elongation by RL, control of flowering, shade avoidance, and end-of-day far-red response (Nagatani et al., 1991, Somers et al., 1991; Childs et al., 1992; Devlin et al., 1992; López-Juez et al., 1992; Reed et al., 1993). Initial studies with *PHYA* -deficient mutants of *Arabidopsis* suggest that phytochrome A plays a major role in perception during the FR-triggered high-irradiance response in de-etiolating seedlings, but plays a minor role in mature plants after de-etiolation under VWL (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993).

The tomato *au* mutant possesses many features of chromophore-deficient *hy*1 and *hy*2 mutants of *Arabidopsis* (Parks and Quail, 1991): etiolated seedlings lack spectrally active phytochrome, possess ill-developed chloroplasts, and arc slow to de-etiolate (Koornneef et al., 1985; Parks et al., 1987; Neuhaus et al., 1993). Etiolated seedlings of *au* show reduction in several phytochrome-mediated responses, such as RL-mediated induction of transcripts of nuclear-encoded plastidic proteins (Sharrock et al., 1988, Oelmüller et al., 1989) and inhibition of hypocotyl elongation (Adamse et al,, 1988) and Chl synthesis (Ken-Dror and Horwitz, 1990). The deficiency of phytochrome in *au* seedlings has been exploited to develop single-cell assays for phytochrome-triggered signal transduction, where microinjection of oat phytochrome A

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Abbreviations: *au, aurea;* BL, blue light; FR, far-red light; *hp, high pigment;* NF, norflurazon; NiR, nitrite reductase; NR, nitrate reductase; PAL, phenylalanine ammonia lyase; RL, red light; VWL, white light; WT, wild type.

into a single cell of an *au* hypocotyl restores photoinduced anthocyanin biosynthesis and chloroplast development in a cell-autonomous fashion (Neuhaus et al., 1993).

The molecular basis of the *au* mutation is not known, but it is not within the *PHYA* gene, because the *PHYA* gene in tomato maps to a chromosomal location distinct from the *au* locus (Sharrock et al., 1988). The etiolated seedlings of *au* possess both phytochrome A and phytochrome B apoproteins, but phytochrome A is spectrally inactive and is present at a level 20% of that in the WT (Sharma et al., 1993). In contrast, light-stable phytochromes (Adamse et al., 1988; López-Juez et al., 1990), such as phytochrome B (Sharma et al., 1993), and photoresponses pertaining to it, such as the end-of-day far-red response, can be detected in green seedlings and mature *au* plants (Adamse et al., 1988; López-Juez et al, 1990; Whitelam and Smith, 1991; Kerckhoffs et al, 1992). It is possible that *au* may be a chromophore mutant similar to the *hy1* and *hy2* mutants of *Arabidopsis* (Parks and Quail, 1991).

In view of the facts that photostable phytochrome and the responses triggered by it are observed in green tissues of *au*, it would be interesting to examine whether the RL-mediated induction of enzyme activities, seen typically during de-etiolation of seedlings, can be observed in etiolated *au* seedlings. Phytochrome induces synthesis of several cytosolic and plastidic enzymes during de-etiolation (Thompson and White, 1991). The photoinduction of enzymes such as NR, NiR (Rajasekhar and Oelmüller, 1987; Rajasekhar et al., 1988; Becker et al., 1992), PAL (Goud et al., 1991), and amylase (Manga and Sharma, 1988; Vally and Sharma, 1991) has been observed in many species. In the present study, we have compared photoinduction of the cytosolic enzymes NR and amylase and the plastidic enzyme NiR in the *au* mutant and WT tomato, in the *hp* mutant, which possesses amplified phytochrome responses (Peters et al, 1992), and in the *au,hp* double mutant. Here, we report that although the phytochrome-deficient *au* mutant retains photoinduction of the cytosolic enzymes NR and amylase, it lacks photoinduction of the plastidic enzyme NiR,

MATERIALS AND METHODS

Growth of Seedlings

Seeds of tomato (*Lycopersicon esculentum* Mill. cv Ailsa Craig) were raised at the Department of Genetics, Wageningen, The Netherlands. Isogenic WT, *hp*, *au*, and *au,hp* obtained as described by Adamse et al. (1989) were used in this study, Seeds were sown in transparent plastic boxes on 20 mL of 0.5% (w/v) agar support containing 5 mM potassium nitrate. Seedlings were grown at 25 ± 1°C in absolute darkness for 96 h and then transferred to continuous RL or WL. In the case of BL pretreatment, 84-h-old dark-grown seedlings were used. The light sources for RL (0.6 W m^{-2}), BL (0.13 W m^{-2}), and FR (3.5 W m^{-2}) were identical to those described by Manga and Sharma (1988).

NR Extraction and Assay

The procedure for the NR (EC 1.6.6.2) assay was essentially followed from Hageman and Reed (1980). Ten hypocotyls or pairs of cotyledons were homogenized in a precooled pestle and mortar on ice in 0.5 mL of extraction buffer (pH 7.8) containing 25 mM potassium phosphate, 1 mM Cys, 5 mM KNO_3, 5 mM EDTA, and 1% (w/v) BSA. The homogenate was centrifuged at 30,000*g* for 30 min at 4°C, and the clear supernatant was used for the assay. The NR assay was performed at 30°C for 1 h in an assay medium containing 200 μL of supernatant, 0.4 mM potassium phosphate buffer (pH 7.5), 0.15 mM KNO_3, and 35.2 μM NADH in a final volume of 0.5 mL. The reaction was terminated with 20 mM zinc acetate, and, after mixing, the tubes were centrifuged for 5 min at 3000*g*. The amount of nitrite formed in the supernatant was estimated by adding 1 mL of *0.2%* (w/v) N-1-naphthyl ethylenediamine hydrochloride and 1 mL of 1% (w/v) sulphanilamide in 3 N HCl, and measuring the A_{540} (Snell and Snell, 1949).

NiR Extraction and Assay

Five hypocotyls or pairs of cotyledons were homogenized at 4°C with 0.5 mL of 0.1 M potassium phosphate buffer (pH 7.5) in a precooled pestle and mortar on ice, the homogenate was centrifuged at 30,000*g* for 30 min at 4°C, and the clear supernatant was used for the assay. The NiR (EC 1.7.7.1) assay was performed at 30°C for 1 h in an assay mixture containing 200 μL of extract, 0.04 mM potassium phosphate buffer (pH 7.5), 0.27 mM methyl viologen, 0.5 mM KNO_2, and 2.5 mM sodium dithionate in a final volume of 0.5 mL (Vega et al, 1980). The reaction was terminated by vigorous vortexing until the blue color disappeared; thereafter, the amount of nitrite utilized was estimated as described above for NR extraction and assay.

Amylase Extraction and Assay

Twenty hypocotyls or pairs of cotyledons were homogenized in a chilled pestle and mortar in 3 mL of buffer containing 0.1 M sodium acetate (pH 5) and 4 mM $CaCl_2$. The homogenate was centrifuged at 30,000*g* for 20 min at 4°C and the clear supernatant was used for assay. The assay was performed in a reaction medium containing 100 mM sodium acetate (pH 5.2), 4 mg/mL amylose, 4 mM $CaCl_2$, 1 mM sodium fluoride, and 500 μL of supernatant in a final volume of 4 mL (Vally and Sharma, 1991). The reaction was conducted for 2 h, and 500-μL aliquots were withdrawn at 1-h intervals. The amount of reducing sugars in the aliquots was estimated by adding an equal volume of dinitrosalicylic acid reagent (Bernfeld, 1955). After mixing, the samples were boiled for 5 min and diluted to 3 ml. with distilled water. The increase in reducing sugars was determined by measuring A_{540} using maltose as standard.

Inhibitors

NF (0.06 mM) and sodium tungstate (2 mM) were used in different experiments. In the experiments where an inhibitor was applied from the time of sowing, the inhibitor was mixed with the agar before solidification. In other experiments, the required amount of an inhibitor was sprayed on the seedlings under a green safelight 2 h before the onset of light irradiation.

RESULTS

Photoinduction of Enzyme Activity

The involvement *oi* phytochrome in the photoinduction ot NR, NiR, and amylase activity in tomato seedlings is confirmed by the RL/FR reversibility experiments. Figure 1 shows that a brief pulse of RL significantly increased NR, NiR, and amylase activities in both cotyledons and hypocotyls of WT and *hp*. The effect of the RL pulse was significantly negated when it was followed bv a FR pulse. In comparison with continuous RL irradiation, the effect *oi* a brief RL pulse on photoinduction *oi* enzymes was weak. In general, the magnitude of photoinduction of enzymes was higher in *hp* than in WT. By contrast, in *au* a brief RL pulse failed to induce activity *of* any *oi* the above enzymes. On the other hand, continuous RL irradiation photoinduced only NR and amylase activities, but not NiR activity, in *au* (Fig. 1) and *au,hp* (data not shown).

Figure 2 shows the time course *oi* NR activity under continuous RL. The continuous RL increased NR activity in cotyledons of WT. *hp,* and *au* after a lag of H h; thereafter, NR activity attained a peak at 24 h before declining gradually. However, at 48 h in WT and *hp,* NR activity was still higher than that in the respective dark controls (Fig. 2A). A comparison of enzyme activities in etiolated seedlings of mutants and WT (Fig.]) reveals that, in general, the basal level of enzyme activities in *au* is less than that in WT, whereas in *hp* it is higher than that in WT. Taking into account the observed

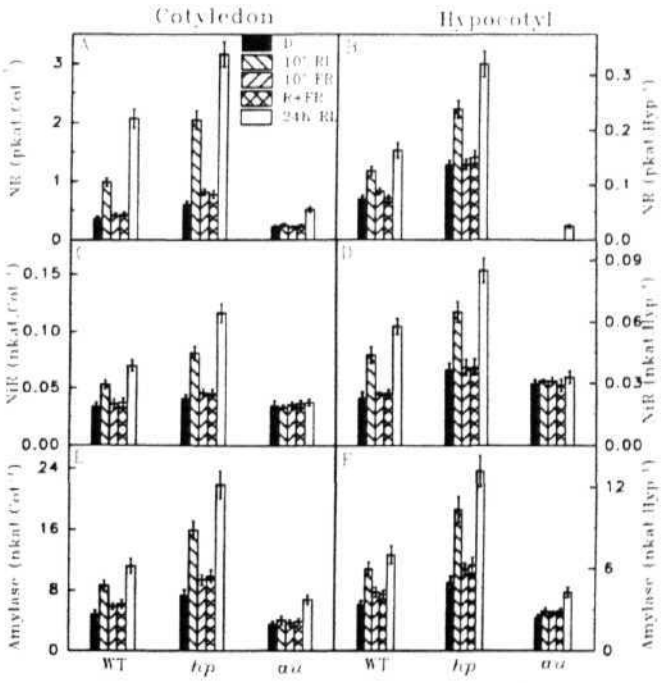

Figure 1. Effect of 10-min RL and FR irradiation on NR (A, B), NiR (C, D), and amylase (E, F) activities in cotyledons (A, C, E) and hypocotyls (B, D, F) of tomato. Seedlings were grown in darkness for 96 h from sowing and then were subjected to continuous RL, brief RL, or FR irradiation as described in the figure. The control seedlings remained in darkness (D). The enzyme activities were estimated at 120 h from sowing.

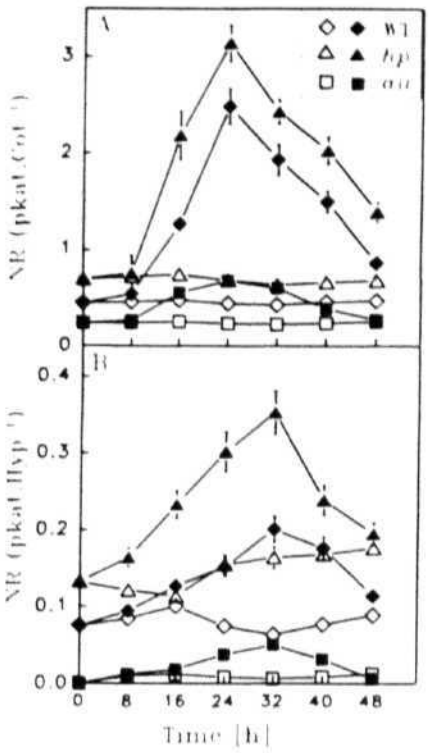

Figure 2. Time course of induction of NR activity in cotyledons (A) and hypocotyls (B) of tomato seedlings. Seedlings were grown up to 96 h from sowing in darkness and were then transferred to continuous RL (closed symbols). The control seedlings were maintained in continuous darkness (open symbols). •, ○, WT; ▲, △, *hp*; ■, □, *au*.

difference in enzymic activities of etiolated mutant and WT seedlings, the relative efficiency of mutants and WT in responding to continuous RL was compared after calculating the fold stimulation of enzyme activity over the respective dark controls. Figure 2A shows that *au,* despite being deficient in phytochrome, retains a significant photoinduction of NR; moreover, the time course of NR increase in *au* cotyledons is similar to that in WT. Although the relative photoinduction of NR activity in *hp* was nearly equal to that in WT, the magnitude of photoinduction of NR in *au* was less than half of that in WT. In a fashion similar to that in the cotyledon, continuous RL induced NR activity in the hypocotyl, except that NR activity attained a peak at about 32 h after the onset of RL (Fig. 2B).

The time course of induction of NiR activity in cotyledons is very similar to that of NR. In WT and *hp* seedlings, RL-induced enhancement of NiR activity peaked at about 24 h (Fig. 3). A higher magnitude of induction of NiR was noticed in cotyledons of *hp* than in WT (Fig. 3A), whereas in hypocotyls the magnitude of NiR induction was nearly equal (Fig. 3B). However, no photoinduction of NiR activity was observed in *au* during the 48 h of continuous RL exposure in either cotyledons or hypocotyls.

The time course of amylase activity under continuous RL (Fig. 4) in mutants and WT was similar to that of NR. After a lag of 8 h, amylase activity peaked at about 24 h, before declining gradually. The time courses of amylase increase in

au cotyledons and hypocotyl were qualitatively similar to that of WT. In the *hp* mutant the amylasc level increased more rapidly than in *au* or WT and attained a significantly higher level at 24 h.

Effect of BL/WL on Enzyme Activity

The effect of BL pretreatment on NR and NiR activity was investigated in both cotyledons and hypocotyls of tomato seedlings (Fig. 5). BL alone showed very little induction of NR activity (except in *hp* cotyledon). There was no significant difference in the photoinduction of NR activity between cotyledons treated with BL before RL and those irradiated with RL alone (Fig. 5A). Similar results were also obtained with hypocotyls (Fig. 5B). In comparison to NR, BL pretreatment induced NiR activity in WT and *hp* seedlings, and a 12-h BL pretreatment alone was as effective as a 24-h RL treatment (Fig. 5, C and D). Moreover, when BL treatment was followed by RL, there was no further increase in the magnitude of NiR induction. By contrast, in *au* and *au,hp* (data not shown) there was absolutely no induction of NiR activity with BL, RL, or BL followed by RL, except in seedlings transferred to continuous WL, where a 60% induction of NiR activity was observed (Fig. 5, C and D).

Effect of Inhibitors

The contribution of de novo synthesis versus activation in photoinduction of NR activity was examined by using sodium

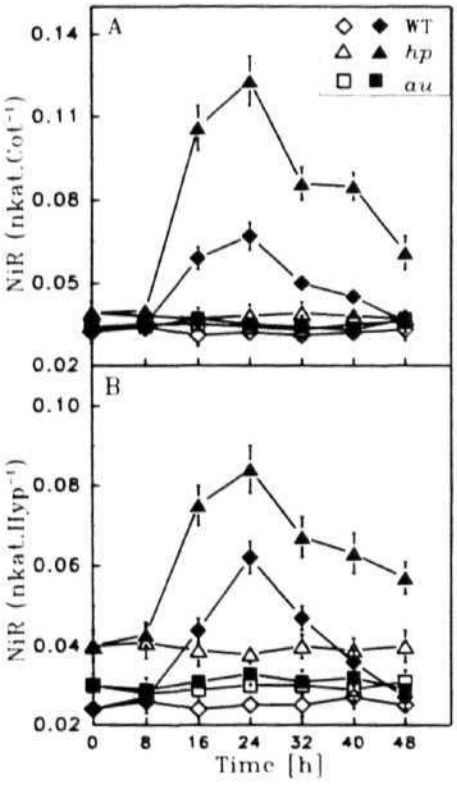

Figure 3. Time course of induction of NiR activity in cotyledons (A) and hypocotyls (B) of tomato seedlings. The experimental conditions and symbols are the same as those described in Figure 2.

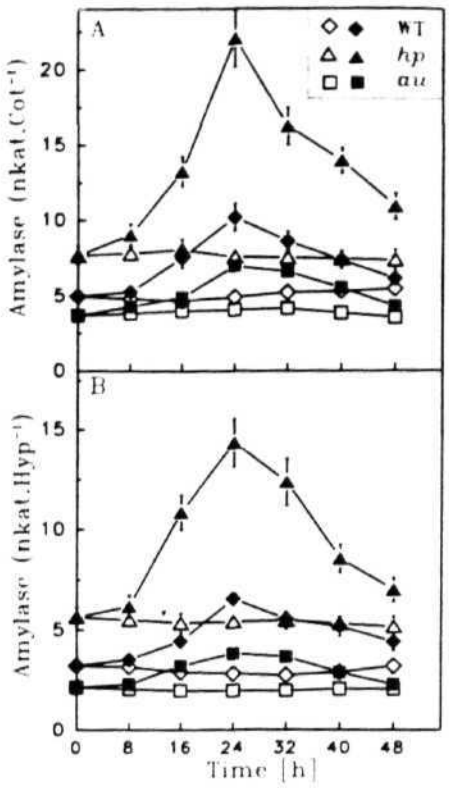

Figure 4. Time course of induction of amylase activity in cotyledons (A) and hypocotyls (B) of tomato seedlings. The experimental conditions and symbols are the same as those described in Figure 2.

rungstate. Tungstate renders newly synthesized NR protein inactive by replacing molybdenum with tungsten as a metal ion (Deng et al., 1989). Since NR and NiR in higher plants are coordinately induced (Faure et al., 1991), the consequence of loss of NR activity on NiR activity was also examined. In seedlings grown in tungstate from the time of sowing, NR activity was less than 5% of both dark- and RL-grown controls of WT and *hp*, and no NR activity could be detected in the *au* mutant (data not shown). In seedlings sprayed with rungstate 2 h before light treatment (Fig. 6), the photoinduction of NR in RL-grown seedlings was totally inhibited. The levels of NR activity in RL-grown tungstate-treated seedlings were close to dark levels in both cotyledons and hypocotyls (Fig. 6, A and B). The tungstate-mediated inhibition of NR had no significant effect on NiR activity in WT and *hp* seedlings (Fig. 6, C and D), but in *au* and *au,hp* (data not shown) seedlings the NiR level dropped below those of the respective controls.

In view of the spatial separation of NR and NiR, their localization being in cytosol and Chloroplast, respectively (Rajasekhar and Oelmüiler, 1987), the effect of NF-mediated loss of functional plastids (Oelmüiler, 1989) on enzyme activity was also studied. In light-grown NF-treated seedlings, NR activity was only partially reduced in mutants and WT, except in the *au* hypocotyl, where NR activity could not be detected in the presence of NF (Fig. 7, A and B). In contrast to NR, photoinduction of NiR was completely abolished by NF treatment in WT and the *hp* mutant. Even in *au*, which lacks

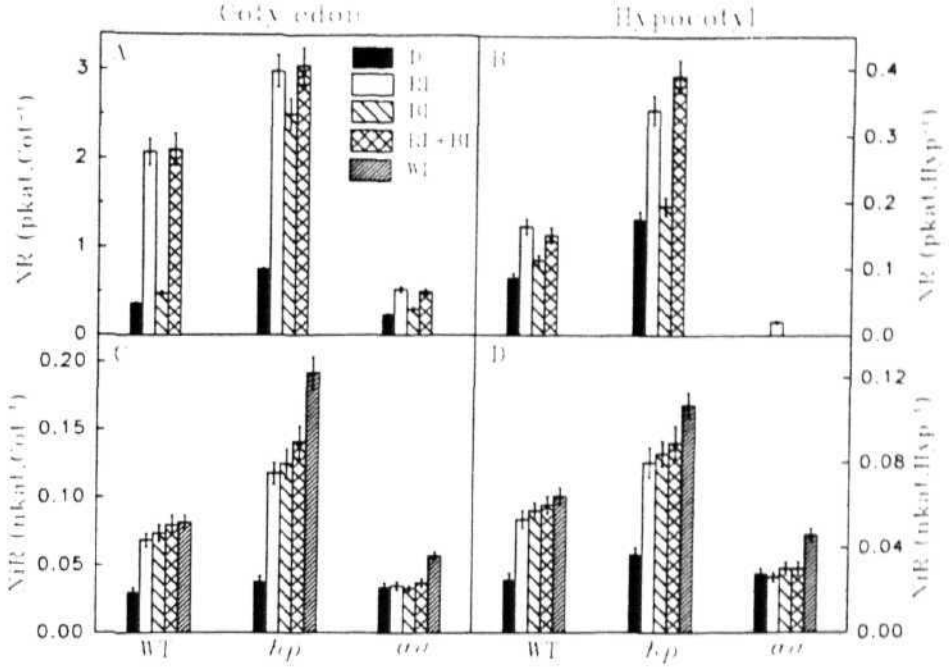

Figure 5. Effect of BL prelreatment on photoinduction of NR (A, B) and NiR (C, *0)* activities in cotyledons (A, C) and hypocotyls (B, D). Seedlings were grown in darkness lor 84 h and were then irradiated with BL tor 12 h. At the end of BL treatment seedlings were either returned to darkness (BL) or were irradiated with continuous RL (RL+BL). In case of the RL and WL treatments, seedlings were grown up to 96 h from sowing in darkness and were then transferred to the appropriate light. Control seedlings were maintained in continuous darkness (D). Enzyme activity was assayed at 120 h from the time of sowing in all samples. In seedlings irradiated with continuous WL, only NiR activity was estimated.

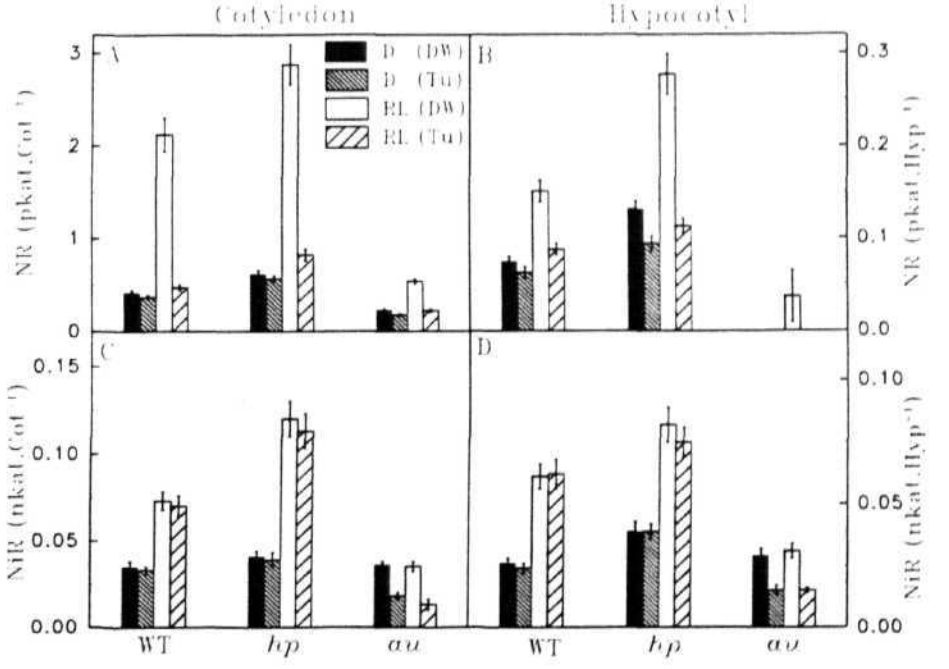

Figure 6. Effect of tungstate (Tu) on photoinduction of NR (A, *B)* and NiR (C, D) activity in cotyledons (A, C) and hypocotyls (B, D). Seedlings were grown in darkness for % h and then a set of seedlings was translerred to continuous RL. Seedlings were sprayed with tungstate (2 mM) under a green safelight 2 h prior to RL exposure. 7he control seedlings were similarly sprayed with distilled water (DW). NR and NiR activity were measured after 24 h of continuous RL.

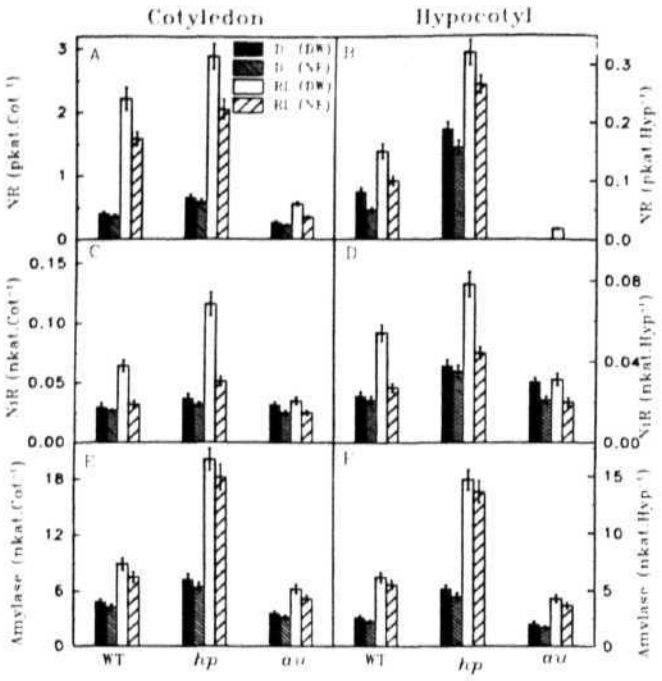

Figure 7. Effect of NF on photoinduction of NR (A, B), NiR (C, D), and amylase (E, F) activity in cotyledons (A, C, E) and hypocotyls (B, D, F). Seedlings were grown on agar support medium containing NF from the time of sowing, whereas controls were grown on medium without NF (DW). Seedlings were grown in darkness for 96 h, and then a set of seedlings was transferred to continuous RL. The control seedlings were maintained in darkness. Enzyme activity was measured after 24 h of continuous RL.

photoinduction of NiR, the level of NiR dropped below that of the respective controls (Fig. 7, C and D). In the case of amylase, NF treatment reduced the photoinduction of amylase in both mutants and WT seedlings only slightly (Fig. 7, E and F).

DISCUSSION

The results obtained with *au* in this study are in accord with the fact that the level of physiologically functional phytochrome is severely depleted in seedlings of this mutant. Although the etiolated *au* seedlings possessed a basal level of amylase, NR, and NiR activity, a brief RL pulse did not stimulate any of these enzymes (Fig. 1). The absence of RL-pulse-mediated enzyme induction in *au* is consistent with previous studies demonstrating that *au* shows little induction of photoresponses with RL pulses (Oelmüller et al., 1989; Oelmüller and Kendrick, 1991; Becker et al., 1992). Although *au* did not respond to brief RL pulses, it showed a stimulation of amylase and NR activity under continuous RL, in a fashion qualitatively similar to WT and *hp* seedlings. The time course of NR and amylase induction in *au* under continuous RL followed profiles similar to those in WT and *hp*, except that the magnitude of enzyme induction in *au* was considerably lower than in WT and *hp* (Figs. 2 and 4). Evidently, barring the absence of NiR photoinduction in *au*, the deficiency of phytochrome in *au*, or amplification of sensitivity to phytochrome in *hp* did not influence the profiles of enzyme induction, such as the duration of lag or the time required to attain peak induction of enzymes. The higher magnitudes of photoinduction of enzymes in *hp* seedlings are in conformity with the observed pleiotropic effect of *hp* mutation on amplification of phytochrome-regulated responses (Peters et al., 1992). By contrast, the observation that continuous RL stimulates amylase, NR (Fig. 1). and PAL activities (Goud et al., 1991) in *au* is at variance with earlier studies where continuous RL-mediated induction of several nuclear transcripts could not be detected in etiolated *au* seedlings (Sharrock et al., 1988; Oelmuller et al,, 1989; Oelmuller and Kendrick, 1991),

The retention of continuous RL-mediated enzyme induction in *au* seedlings, despite severe reduction in the spectrally active phytochrome A level, indicates that *au* has a residual active phytochrome pool that regulates the above responses. Physiological experiments have shown that mature plants of *au* retain the end-of-day far-red response (Adamse et al., 1988; López-Juez et al., 1990) and shade-avoidance reactions (Whitelam and Smith, 1991; Kerckhoffs et al., 1992), which are assumed to be mediated by a photostable phytochrome. Since mutants deficient in phytochrome B apoprotein (López-Juez et al., 1992; Reed et al., 1992, 1993) lack the above photoresponses, it is assumed that phytochrome B is functional in mature *au* plants. Moreover, the elution profile of spectrally active phytochrome from green leaves of *au* was similar to that of phytochrome B, indicating that in mature *au* plants phytochrome B is spectrally active (Sharma et al., 1993). Considering the existence of spectrally active phytochrome in green *au* plants and the retention of phytochrome-triggered responses in *au* seedlings, it is plausible that the residual phytochrome pool of *au* seedlings may consist of photostable phytochromes. Since phytochrome in tomato is encoded by at least three genes (Hauser and Pratt, 1990), this pool may consist of one or more active phytochrome species. However, the relative proportions and functional contributions of phytochrome species constituting the phytochrome pool in *au* are not known.

Although it has been tacitly assumed that defective photoregulation in *au* arises from phytochrome deficiency, the pleiotropic nature of the *au* mutation cannot be ignored. Although it has been suggested that *au* is a phytochrome A-deficient mutant (Sharma et al., 1993), it is now apparent that the phytochrome A null mutant of *Arabidopsis* grown under WL displays a phenotype almost indistinguishable from WT (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). In contrast, *au* plants grown under normal daylight differ from WT, being pale green in color (López-Juez et al., 1990; Becker et al., 1992) and, unlike WT, possess an *agranal* Chloroplast with a reduced number of thylakoid membranes (Koornneef et al., 1985). Such a pleiotropic effect on Chloroplast development may complicate experimental interpretations. For instance, it has been shown that expression of nuclear genes like *CAB* and *RBCS* that encode plastidic proteins is closely associated with Chloroplast development (Susek et al., 1993). In the present study, although photoinduction of cytosolic enzymes such as amylase and NR (Fig. 1) is present in *au*, a similar induction of plastidic proteins like NiR (Fig. 3) and of mRNA levels for other

plastidic proteins (Sharrock et al., 1988; Oelmuller and Kendrick, 1991) is not seen. The absence of photoinduction of NiR in *au*, even under continuous RL, is an intriguing observation. Becker et al. (1992) showed that although a brief RL pulse failed to elevate NiR and NR transcript levels in *au*, continuous RL elevated both NR and NiR transcripts. Whereas RL-mediated increase in NR transcript level (Becker et al., 1992) stimulated de novo synthesis of NR in *au* (Fig. 6), as revealed by tungstate-mediated inactivation of newly synthesized NR molecules (Deng et al., 1989), a similar increase in NiR transcript level in *au* is not accompanied by a stimulation in NiR enzyme level.

It is likely that the observed discrepancy between the RL-mediated increase in NiR transcript level and the absence of photoinduction of NiR activity may arise from a block in Chloroplast development in *au*. Although phytochrome induction of NiR transcript (Becker et al., 1992) may not be tightly linked with Chloroplast differentiation, because it is a plastidic enzyme, the expression of NiR activity is likely to be dependent on Chloroplast development. Neuhaus et al. (1993) have unequivocally shown that in hypocotyl cells of etiolated *au* seedlings, plastid development is arrested at the level of proplastids, which do not even differentiate into etioplasts. Moreover, these proplastids do not transform to chloroplasts, even after a 48-h exposure to WL. It is plausible that the above delay in Chloroplast development in *au* may in some way be responsible for the absence of photoinduction of NiR. The observation that NF-induced photooxidation of chloroplasts drastically reduces the NiR level in WT and *hp* indicates that Chloroplast integrity is essential for photostimulation of NiR activity.

Since *au* mutants survive despite being deficient in phytochrome and having impaired Chloroplast biogenesis, it is possible that a co-action by another photoreceptor during deetiolation may alleviate the adverse effects of phytochrome deficiency. For example, BL pretreatment of *au* restores phytochrome-mediated induction of nuclear transcripts encoding plastidic proteins (Oelmuller and Kendnck, 1991). Although NiR is a nuclear-encoded plastidic protein, a BL pretreatment of *au* did not induce NiR activity, whereas BL largely replaced RL-mediated NR and NiR induction in *hp* and WT. Only when *au* seedlings were exposed to continuous WL could a reduced level of NiR photoinduction be observed (Fig. 5). Likewise, photoinduction of *rbcs* transcripts in *au* was seen only under continuous WL (Sharrock et al., 1988). Probably a simultaneous operation of BL photoreceptor and residual phytochrome under continuous WL restores the photoinduction of NiR by stimulating Chloroplast development in *au* seedlings.

The above discussion highlights the fact that although *au* retains photoinduction of enzymes that are possibly regulated by residual phytochrome, deficiency in NiR photoinduction may ensue from defective Chloroplast development under RL. In view of the pleiotropic effects of the *au* mutation, a further biochemical and genetic analysis of this mutant is required.

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