GENETIC AND PHYSIOLOGICAL REGULATION OF PHOTOTROPIC RESPONSE DURING DE-ETIOLATION IN TOMATO *(Lycopersicon esculentum* L.) SEEDLINGS

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BY

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CERTIFICATE

This is to certify that the thesis entitled **"Genetic and physiological regulation of phototropic response during de-etiolation in tomato *(Lycopersicon esculentum* L.) seedlings"** is based on the results of the work done by **Mr. A. Srinivas** 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 dissertation 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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LIST OF ABBREVIATIONS AND SYMBOLS

A	absorbance
au	*aurea* mutant of tomato
BL	blue light
BSA	bovine serum albumin
cBL	continuous blue light
°C	degree Celsius
chl	chlorophyll
cv	cultivar
D	dark (ness)
DCPIP	di-chloro-phenol-indo-phenol
EMS	ehyl-methane-sulfonate
EODFR	end-of-day FR response
FR	far-red light
fri	*f*ar-*r*ed-*i*nsensitive mutant of tomato
HIR	high irradiance response
hp-1	*high pigment-1* mutant of tomato
HP-1	product of *HP* gene
LFR	low fluence response
M|.,	1-x no. of generation after mutagenesis
Pfr	far-red light absorbing form of phytochrome
phy	gene coding for phytochrome
PHY	phytochrome apoprotein
phy	phytochrome holoprotein
Pr	red light absorbing form of phytochrome
RL	red light
rpm	revolutions per minute
s	seconds
SE	standard error
tri	*t*emporarily-*r*ed light-*i*nsensitive mutant of tomato
VLFR	very low fluence response
WL	white light
WT	wild type
h	hour
NF	norflurazon
kDa	kilo Dalton
UV-A	ultra-violet light (315-400 nm)
V/v	volume/volume
W/v	weigh/volume

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CHAPTER 1

INTRODUCTION

Higher plants have evolved complex strategies to adapt to environmental changes by inducing or altering physiological and developmental responses. The interacting environmental cues and physiological changes comprise of perception of stimuli by different receptors, activation of complex intracellular signaling pathways following a change in the pattern of gene expression. Information about the cellular and molecular mechanisms of signal perception and transduction is gradually accumulating and are in their infancy (Bowler and Chua, 1994). However, it is becoming increasingly clear that several environmental factors are involved in several different pathways and some transduction components playing a common role in several pathways, with the potential for overlap and interaction of signal transduction pathways. As a result many different environmental signals are also known to elicit the same physiological response indicating further complexity of transduction mechanisms. Hence, to gain a full understanding of signal transduction in plant cells, it has become important to define the interactions between signaling pathways as well as to identify their primary components.

Among the environmental factors, light provides informational signal that plants use to adapt and optimize the growth and development through a process known as photomorphogenesis (Kendrick and Kronenberg, 1994). Examples of such photomorphogenic responses are observed during every stage of life cycle of plant which include the promotion of seed germination, de-etiolation of dark-grown seedlings, phototropism of stems and leaves, expansion of leaves, sensitivity of gravitropism, induction of flowering and senescence. In addition, in a shaded plant, the light environment transmitted and reflected from neighboring plants also induces photomorphogenic response known as shade avoidance response. Many of these light-controlled

developments are triggered by alteration in gene expression through the regulation of specific genes in defined cell types and developmental stages.

To regulate these multifarious and complicated processes plants have evolved distinct photoreceptors. At least three major classes of photoreceptors are involved in the perception of different regions of light spectrum namely red/far-red reversible phytochromes, blue/UV-A photoreceptors and UV-B photoreceptor(s) (Kendrick and Kronenberg, 1994). Although there is extensive body of data for existence of multiple types of photoreceptors, it has only recently became apparent that phytochromes and blue light photoreceptors are encoded by multiple divergent genes. Each of these photoreceptors can regulate multiple independent photoresponses. For example, phytochromes plays a role in seed germination, de-etiolation, shade avoidance and the induction of flowering (Quail et al., 1995; Smith, 1995; Chory et al., 1996) while blue light photoreceptors mediate responses such as hypocotyl growth inhibition and phototropism (Kaufman, 1993; Short and Briggs, 1994; Ahmad and Cashmore, 1996).

Moreover, many of the photoresponses also require co-action of more than one photoreceptor, for example, phytochromes and blue light photoreceptor(s) co-act in the regulation of hypocotyl growth, anthocyanin synthesis, enhancement of phototropism and gene expression (Casal, 2000). Both phytochromes and blue/UV-A photoreceptors can elicit the same response, as in the case of hypocotyl growth inhibition and anthocyanin synthesis or both receptors can act in interdependent way to elicit the same response as in the case of phototropism. The regulatory roles of different photoreceptors and their interactions have been defined largely through

physiological studies, however, the potential of this approach is limited and it cannot be used to understand the action and function of different members of photoreceptors, the mechanism of their co-action and transduction pathways triggered by them.

In recent years, application of genetic approaches have made significant advances for characterizing the components of light signal transduction chain through analysis of photomorphogenic mutants (von Arnim and Deng, 1996; Fankhauser and Chory, 1997). Several mutants isolated are affected in the genes involved in sensing and responding to light. The characterization of these mutants by genetic and physiological assays has divulged the nature and mechanism of action of various photoreceptors or putative signal elements (Neuhaus et al., 1993; Bowler et al., 1994). In many cases where virtually nothing was known about the nature of gene products, the molecular genetic characterization of loci by cloning and sequencing of genes have provided information about the possible molecular and biochemical functions (Ahmad and Cashmore, 1993; Pepper et al., 1994; Deng, 1994). Notwithstanding the fact that the many aspects of early light induced changes leading to the activation of gene expression are now known in great detail, however, the dissection of specific mechanisms of light-signal transduction of different photomorphogenic remains poorly understood. Furthermore, it is clear that additional mutants will be necessary to define the number and complexity of the pathway(s) involved in photomorphogenic responses.

Phototropism, a growth response to unilateral or asymmetric light, is an important component of plant photomorphogenic response to the directional changes in light environment. Action spectra for the phototropism have

indicated that it is mediated only by a blue/UV-A light photoreceptor. Recently with the advent of genetic studies on phototropism, a putative blue light photoreceptor controlling phototropism has been identified (Short and Briggs, 1994; Liscum and Briggs, 1995; Huala *et al.,* 1997) and it has been shown to be a dichromophoric flavoprotein (Christie *et al.,* 1999). While only blue/UV-A photoreceptor is effective in inducing phototropism, pre-irradiation with red/far-red light activating phytochrome photoreceptor is also known to enhance the phototropic response to subsequent blue light treatment, which led to the conclusion that phytochrome might modulate but not initiate the phototropic response. In contrast, from the available genetic studies with phytochrome deficient mutants (Janoudi et al., 1997) and physiological experiments (Liu and Iino, 1997) it has been reported that the phytochromes are also required for full expression of phototropic response. However, the mode of co-action between the phytochromes and blue light photoreceptor is still not known clearly. Furthermore, we have none or only very few information about most steps and the sequence of action and relative participation of the photoreceptors in stimulating phototropic signal transduction pathway and to bring final growth change.

The present study was undertaken 1. to investigate the role of phytochromes in blue light-induced phototropism and red/far-red light mediated enhancement of phototropism 2. to identify additional constituents involved in transduction pathway of phototropism through isolation and characterization of mutants. The search for components in the signal transduction chain that couple the photoreceptor to cellular systems is aided by the use of a model system. The tomato plant has been one of such suitable plant materials to study the light-induced signal transduction (Kendrick et al., 1997).

The phytochrome gene family in tomato has been characterized during recent years (Pratt et al., 1997). Several photomorphogenic mutants affecting phytochrome functions and signal transduction have been isolated (Kendrick et al., 1997). This enables to further study these mutants to reveal the individual functions of phytochromes and their co-action with blue/UV-A photoreceptors, if any, in phototropism. In addition, tomato is an ideal genetic system and can be used to identify mutants affected in phototropism. It is also an ideal system for positional cloning of unidentified genes because of the relatively small genome size with mostly single copy genes and little repetitive DNA. A detailed genetic and RFLP maps has been generated (Tanksley et al., 1992) and markers have been made available for mapping studies. The ease of transformation and targeted transposon tagging also facilitates to isolate genes and provide a starting point for signal transduction mechanism of phototropism in this species.

In this study, therefore, an attempt was made to elucidate the regulation of phototropism using tomato as a model system. The mode of co-action of phytochrome and blue light photoreceptor in regulating phototropic response of tomato seedlings was characterized during the process of de-etiolation. Furthermore, the role of phytochrome in inducing phototropic response and the enhancement were investigated in wild type and mutants by using phytochrome specific mutants. The results demonstrate that the induction and enhancement of phototropic adaptation in tomato is regulated by A-type phytochrome, although there is some redundancy with other members of phytochrome gene family. The identification of *hp-1* mutation has been helpful in defining a potentially important signal transduction pathway involved in light-regulated seedling development in tomato. Its involvement in the phytochrome

transduction pathway was proposed by Peters et al., (1992) and it has been suggested that it acts as negative regulator in the pathway. The possible involvement of *HP-1* gene product in phototropic transduction pathway was examined in its double mutants with phytochrome deficient mutants. The results indicate that *HP-1* gene product also acts as a negative regulator in the phototropic response of tomato seedlings. In order to identify additional components involved in photbtropism, mutants were isolated that are non-phototropic to light. The genetic and physiological characterization of two non-phototropic mutant lines obtained indicates that the mutations affect specifically phototropism and act early in the pathway of signal transduction.

CHAPTER 2

REVIEW OF LITERATURE

Since light provides the energy source for photosynthesis plants have evolved mechanisms for sensing light parameters such as its presence, the direction, magnitude, quality and photoperiod length (Mancinelli, 1994) to adapt to the light environment. The adaptive responses are seen throughout the life cycle of plant, which include growth changes such as phototropism and developmental changes like de-etiolation and floral induction (Kendrick and Kronenberg, 1994). The physiological study of these responses has defined the basics of how plants detect and respond to changes in light environment but have not led to a detailed understanding of them. In recent years, the studies have been focused to elucidate the mechanisms of detecting light signals by the photoreceptors and transducing the signals into the appropriate intracellular and molecular events (Fairchild and Quail, 1998; Deng and Quail, 1999; Briggs and Huala, 1999; Lin, 2000; Smith, 2000).

LIGHT PERCEPTION - PHOTORECEPTORS AND FUNCTIONS

2.1 DE-ETIOLATION

Among the responses induced by light, the de-etiolation of seedling, is one of the most important phenomena in plant growth and development (Deng, 1994). Seedlings upon germination in darkness show developmentally repressed process known as skotomorphogenesis and have elongated hypocotyls, closed apical hooks and unexpanded cotyledons. There is little or no expression of photosynthetic genes encoded by nuclear and plastid genomes and plastids develop into etioplast that possess no chlorophyll and are not photosynthetically competent. In the presence of light, however, the seedlings undergo a developmental process known as photomorphogenesis, which involves inhibition of hypocotyl growth, opened apical hook, expanded cotyledons, transformation of etioplast to chloroplast, anthocyanin induction and stimulation of a large number different genes. This process has been used

as a model system to study the mechanisms of light signal transduction leading to photomorphogenesis in plants (Deng, 1994).

2.2 PHOTORECEPTORS

The processing of light information by a plant in different photomorphogenic responses involves the function of photoreceptors. These photoreceptors are thought to comprise three classes viz., the red/far-red photoreceptors-phytochromes, the blue/UV-A and UV-B photoreceptors (Kendrick and Kronenberg, 1994), each of which is able to detect light of particular wavelengths. Of these phytochromes have been most thoroughly studied and in recent years blue/UV-A photoreceptors have been characterized (Ahmad and Cashmore, 1993; Haula et al., 1997; Briggs and Huala, 1999; Lin, 2000), however, the nature of UV-B photoreceptors remains elusive although evidences exist for a separate receptor (Young et al., 1992). The environmental light signals perceived by these three classes of photoreceptors apparently contribute to the photomorphogenic development.

2.3 PHYTOCHROME RESPONSES

In the red/far-red region of the spectrum, plants sense light with the regulatory photoreceptor phytochromes (Kendrick and Kronenberg, 1994; Smith, 2000). Phytochromes control responses such as seed germination, seedling de-etiolation, gene expression, chloroplast differentiation, floral induction, fruit ripening and senescence (Smith, 1995). The phytochromes are also responsible for shade-avoidance response (Smith, 1995) and end-of-day far-red responses (Furuya, 1993). In addition, phytochromes interact with the gravity sensing mechanism to control gravitropism (Gaiser and Lomax, 1993;

Parks *et al.,* 1996) and enhancement of phototropism (Parks et al., 1996; Janoudi et al., 1997).

2.4 PHYTOCHROME GENE FAMILY

The large diversity of phytochrome-mediated responses is due to the expression of multiple phytochrome species in different plants (Furuya, 1993; Chory, 1994; Pratt et al., 1995; Smith, 1995). Recent genetic and biochemical evidence has shown that a small family of genes in all plant species so far studied encodes the phytochrome apoproteins. The exact number of genes coding for phytochrome is ambiguous. The cDNA sequences derived from *Arbaidopsis* has revealed five phytochrome coding regions and designated them PHY A, B, C, D and E (Shamrock and Quail, 1989; Quail, 1991;Clack *et al.,* 1994). Based upon partial sequences of genomic DNA fragments obtained by the PCR, five tomato PHY genes have been identified (Hauser *et al.,* 1995; Pratt, 1995) and these genes were classified as PHY A, PHYB1, PHYB2, PHYE and PHYF. The five *Arabidopsis* PHY genes are expressed at both the mRNA (Clack *et al,* 1994) and protein (Somers *et al.,* 1991) levels, yielding products of the sizes predicted from gene and cDNA sequences. These gene products are present though out most stages of plant development and in most plant organs (Hauser *et al.,* 1998).

Phylogenetic analysis of the phytochrome genes of higher plants suggests that duplication of an ancestral gene at about the time of origin of the seed plants led to the divergence of two lineages, one giving rise to the phyA and phyC homologs and the other giving rise to phyB, phyD, and phyE homologs. Subsequent duplications are proposed to have occurred near the time of the origin of flowering plants (Mathews and Sharrock, 1997). In *Arabidopsis* the phyB and phyD proteins share approximately 80% amino acid

sequence identity and are thought to result from a gene duplication in a recent progenitor of the Cruciferae (Mathews and Sharrock, 1997). The phyB and phyD proteins are more closely related to phyE than they are to either the phyA or phyC proteins.

2.5 TYPES OF PHYTOCHROMES

The proteins encoded by the phytochrome genes are differentiated to at least two different types. The type I is highly abundant in dark-grown seedlings and becomes severely depleted once exposed to light whereas type II is stable and is present at relatively similar levels in dark-grown and light-grown seedlings. The type I phytochromes (Furuya 1993) are mainly involved in de-etiolation processes of etiolated plants, while the type II phytochromes (Furuya 1993) in light-grown plants. The PHYA is the abundant light-labile phytochrome in dark grown seedlings and represents species defined by type I phytochrome (Furuya 1993). The light-stable PHYB shows a longer half-life and is the predominant phytochrome species in light-grown tissue, which represents type II along with remaining members of the gene family. Moreover, PHYA and PHYB are immunologically distinct and differ slightly in their molecular mass. In dicotyledons plants, both proteins possess indistinguishable spectral properties (Wagner *et al.*, 1991), whereas PHYB from oat is spectroscopically distinct from PHYA (Tokuhisa and Quail, 1989; Pratt *et ai*, 1991).

2.6 PHYTOCHROME - MOLECULAR PROPERTIES

The molecular properties of phytochrome have been determined most extensively for the abundant protein species purified from dark grown oat seedlings (Virestra and Quail, 1983). The phytochrome is a chromoprotein

existing *in vitro,* and possibly *in vivo* as well, as active homodimer (Cherry and Vierstra, 1994) with a monomeric molecular mass of 125 kDa. Each monomer of the phytochrome molecule folds into two major domains separated by a protease-sensitive hinge region: 1. the N-terminal chromophore domain of about 60-70 kDa, which is highly conserved in all phytochrome species and to which the tetrapyrole chromophore is covalently attached that mediates light perception, and 2. more divergent C-terminal domain of about 30-50 kDa with one or more dimerization domains (Rudiger and Thummler, 1991; Quail, 1991; Furuya, 1993; Furuya and Song, 1994).

The chromophore of phytochrome, phytochromobilin, is synthesized in the plastid from 5-aminolevulinic acid (ALA) via the heme branch of the tetrapyrrole pathway (Terry et al., 1993), and evidence from reconstitution studies using recombinant phytochromes indicate that different phytochromes use the same chromophore. Phytochrome is assembled in its inactive, Pr form, and current evidence indicate that assembly *in vivo* is autocatalytic and takes place in cytoplasm (Lagarias and Lagarias, 1989; Terry *et al,* 1993). The absorbance of light by the chromophore, can lead to a Z/E isomerization of the tetrapyrrole (Rudiger *et al,* 1983; Farrens *et al,* 1989). This photoreversible process mediates the conformation change of the photoreceptor from the physiological inactive Pr to the active Pfr. Following conversion to the active, Pfr form, phytochrome activates signal transduction pathways that lead to changes in gene expression (Gilamartin *et al,* 1990; Thompson and White, 1991) which underlie wide range of developmental responses. However, the

On the contrary, recent studies of transgenic overexpressing phytochrome genes in *Arabidopsis* have indicated that the photosensory specificities exhibited by phytochromes are governed by sequences in N-terminal domain of their respective polypetides (Boylan and Quail, 1989, 1991; Kay *et al,* 1989; Keller *et al,* 1989; Cherry *et al,* 1991; Wagner *et al,* 1991; McCormac *et al.* 1993; Quail et al, 1995; Xu et al., 1995; Wagner and Quail, 1995). The C-terminal region is responsible for regulatory capacity of the photoreceptors and contains a proximal PAS homology domain that is delimited by two direct repeats showing sequence similarity to the repeats that define PAS domains (Lagarias *et al,* 1995; Kay, 1997) which is a hotspot for missense mutations that affect phytochrome function (Quail *et al.,* 1995) and a distal histidine kinase homology domain that shows sequence similarity with transmitter modules of bacterial protein histidine kinases two-component systems (Schneider-Poetsch, 1992). The latter constitute the environmental sensor proteins of bacteria, suggesting that phytochrome acts as a light-regulated protein kinase (Schneider-Poetsch et al., 1991; Thummler *et al.,* 1995a). This proposal has received strong support due to the recent identification of genes homologous to phytochrome in cyanobacteria which also exhibit the canonical motifs of the catalytic domain of histidine kinases within their C-terminal regions (Kaneko *et al.,* 1996; Kehoe and Grossman, 1996).

2.7 PHYTOCHROME REGULATION OF GENE EXPRESSION

Phytochrome affects the expression of several different genes by affecting the abundance of mRNAs. In many photomorphogenic responses the expression of some genes is induced by irradiation, these include the *rbc* S genes encoding small subunit of ribulose bisphosphate carboxylase/oxygenase

and *cab* genes encoding chlorphyll a/b binding light-harvesting proteins (Silverthrone and Tobin, 1984), chloroplastic *Gln* synthetase (Edwards and Coruzzi, 1989) and *Fd* (Dobres et al., 1987), and others (Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995). On the other hand some genes, however, become repressed when plants are exposed to light. These include *phyA* gene encoding phytochrome A (Colbert et al., 1983), *Asn* synthease (Tsai and Coruzzi, 1990), and *pcr* genes encoding protochlorophyllide oxidoreductase (Forreiter et al., 1990).

In oat and rice the reduced PHYA mRNA levels in red or far-red light largely result from a strong and rapid decrease in transcription (Lissemore and Quail, 1988; Kay *et al.,* 1989) which is most likely regulated via the phyA signal transduction pathway (Quail, 1994). Short periods of red light do not detectably reduce PHYA mRNA levels in tomato and *Arabidopsis,* although continuous white light is effective (Sharrock and Quail, 1989; Somers *et al.,* 1991; Quail, 1994). In contrast, PHYB transcript accumulates in rice, potato and *Arabidopsis* to similar levels, regardless of light treatment (Sharock and Quail, 1989; Dehesh *et al.,* 1991; Somers *et al.,* 1991; Heyer and Gatz, 1992; Clack *et al.,* 1994). Consistent with this idea, red light treatment of dark-grown *Arabidopsis* seedlings has little effect on PHYB polypetide levels (Somers *et al.,* 1991).

Several promoters of *PHYA, rbcS* and *Lhcb* gene families have been examined to characterize the ***cis*** elements specifically necessary for mediation of phytochrome regulation (Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995). For the *PHYA* gene a **cis** element involved in repression of activity was found in a 10-bp fragment of promoter (Bruce *et al.,* 1991). A 166-bp fragment

of the pea rbcS-3A promoter was found to be involved in phytochrome regulation (Gilmartin and Chua, 1990). The *Arabidopsis Lhcb1*1* gene was found to retain phytochrome regulation and circadian responsiveness in a promoter fragment from −111 to -33 (Anderson *et al.*, 1994), and mutations in the region from -74 to -58, which contains multiple GAT A elements, abolished phytochrome responsiveness (Anderson and Kay, 1995). For the *Arabidopsis Lhcb1*3* gene, sequences downstream of −183 were able to confer phytochrome responsiveness (Sun *et al.*, 1993).

In some instances, it has been shown the *cis*-elements involved in the light responsiveness of the genes are occupied by protein factors. A promoter region of *Lhcb1*3* gene from -138 to -99 is bound *in vitro* by a protein factor, CA-1 (Sun *et al.*, 1993), and this region is necessary for phytochrome regulation and contains an element involved in maintaining a high level of transcription (Kenigsbuch and Tobin, 1995). In the case of the *cabE* gene, many different factors, including the G-box binding factor (GBF), GA-1, GC-1, AT-1, and GT-1, have been shown to bind to different promoter elements, probably mediating light responsiveness through protein-protein interactions (Schindler *et al.*, 1990, 1992a, 1992c; Dehesh *et al.*, 1992; Gilmartin *et al.*, 1992; Perisic and Lam, 1992).

2.8 PHYTOCHROME MUTANTS

Two classes of phytochrome-deficient mutants have been identified viz., mutations affecting specific phytochromes and mutants showing general phytochrome-deficiency (Kendrick and Nagatani, 1991; Reed *et al.*, 1992; Chory, 1993; Koornneef and Kendrick, 1994; Liscum and Hangarter, 1994; Whitelam and Harberd, 1994). The phytochrome A specific mutants are blind

to FR, but respond to R. Mutants deficient in phyA are known in several plant species which have been named as long hypocotyl (*hy-8*: Parks and Quail 1993), far-red elongated (*fre1*: Nagatani *et ai,* 1993) and far-red long hypocotyl (*fhy2*: Whitelam *et ai,* 1993) of *Arabidopsis*, the far red insensitive mutant of tomato (*fri*; Van Tuinen *et ai,* 1995a), far-red unresponsive mutant of pea (*fun1*: Weller *et ai,* 1995b). These mutations have been shown to represent in the PHYA gene of independent alleles *of Arabidopsis* (Dehesh *et al.,* 1993; Whitelam *et ai,* 1993) and due to splicing of mRNA transcript in tomato (Lazarova *et ah,* 1998).

The phytochrome B specific mutants are elongated compared to wild type under red light (R) but not far-red light (FR). In addition, the phyB mutants exhibit reduced cotyledon size, elongated petioles and leaves, stronger apical dominance, and early flowering (Reed *et al.,* 1994). Mutants deficient in phyB are known in the *Arabidopsis* long hypocotyl *(hy-3 = phyB;* Koornneef *et al.,* 1980; Nagatani et al, 1993; Somers *et al.,* 1991; Reed *et ai,* 1993), the temporarily red light insensitive mutant of tomato *{tli;* van Tuinen *et ai,* 1995 b), the long hypocotyl mutant of cucumber (*lh*; Lopez-Juez *et ai,* 1992), the *lv* mutant of pea (Weller *et ai,* 1995a), the elongated internode of *Brassica rapa {ein;* Devlin *et ai,* 1992) and the maturity mutant of *Sorghum {ma3R;* Childs *et ai,* 1992). All these mutants have been shown to be deficient in immunochemically detectable phyB (Devlin *et ai,* 1992; Reed *et ai,* 1993; Weller *et al.,* 1995a; Kerckhoffs *et ai,* 1996). For the *phyB* mutant of *Arabidopsis,* the *ma3R* mutant of *Sorghum* and the *ein* mutant of *B.rapa* it has been directly established that the observed phyB deficiency results from a mutation within the *PHYB* gene (Reed *et ai,* 1993; Devlin *et ai,* 1997; Childs *et al.,* 1996).

In addition to these specific phytochrome mutants a second class of phytochrome-deficient mutants have an elongated phenotype under both R and FR corresponding to the absence of both phy A and phyB activities (Koomneef *et al,* 1980; Koornneef *et al,* 1985; Weller *et al,* 1996, 1997). The absence of these responses is correlated to a loss of all or most of the spectrophotometrically detectable phytochrome in dark-grown seedlings, indicating that these mutants are lacking the chromophore of phytochrome. The long hypocotyl *hy1* and *hy2* mutants of *Arabidopsis* (Koomneef *et al,* 1980; Chory *et al,* 1989a; Parks and Quail, 1991), *aurea* (*au*) and *yellow green-2* (*yg-2*) mutants of tomato (Koornnef *et al,* 1985; Terry and Kendrick, 1996; Van Tuinen *et al,* 1996), phytochrome chromophore deficient (*pcd1* and *pcd2*) mutants of pea (Weller *et al,* 1996, 1997) and partly etiolated in white light (*pew1* and *pew2)* mutants of tobacco (Kraepiel *et al,* 1994) are known to be phytochrome chromophore deficient mutants.

Recent analysis has shown that a specific lesion in chromophore deficient mutants is a consequence of a lesion in chromophore synthesis (Weller *et al,* 1996, 1997; Terry and Kendrick 1996). The *hy-1,* and *hy-2* of *Arabidopsis* contain lesions that result in deficiencies in the photochemically functional phytochrome (Koomneef *et al,* 1980; Chory *et al,* 1989a; Parks *et al,* 1989) and affect the synthesis of the phytochrome tetrapyrrole chromophore, since functional phytochrome can be produced by providing these mutants with chromophore precursor biliverdin Ixa or phycocyanobilin (Parks and Quail, 1991). *HY1* gene has recently been cloned and appears to encode a heme oxygenase (Davis *et al,* 1999). The *pewl* mutant of *Nicotiana plumbaginifolia* (Kraepiel *et al,* 1994) can also be rescued by feeding chromophore precursor. The *pew2* mutant, however, could not be rescued by

feeding biliverdin, which is also the case of *au* mutant of tomato. The *pcdl* and *yg-2* mutants are unable to synthesize BV IX from heme (Weller *et al,* 1996; Terry & Kendrick 1996) and are deficient in phytochromobilin synthase activity (Terry & Kendrick 1996; Weller *et al,* 1997). The *hy6* mutant has also been proposed to be a chromophore-related mutant, defective in either the biosynthesis or attachment of the chromophore to the phytochrome apoprotein (Chory *et al,* 1989a; Chory 1992).

In all chromophore-deficient mutants, since all phytochromes probably use the same chromophore, it is possible that all phytochromes are deficient in biological function in these mutants and display extreme pleiotropic phenotypes (Koornneef *et al,* 1980; Chory *et al.,* 1989a). The amount of PHYA which accumulates varies considerably between species ranging from estimates of about 25% of wild type levels for the *au* (Sharma *et al,* 1993) and *yg-2* (van Tuinen *et al,* 1996a) mutants to close to 100% in *pcdl* (Weller *et al,* 1996) and *pew1* (Kraepiel *et al,* 1994). The *hy* mutants (Chory *et al,* 1989a; Parks *et al,* 1989), *pcd2* (Weller *et al,* 1997) and *pew 2* (Kraepeil *et al,* 1994) all have PHYA levels somewhere between these two extremes. In contrast, PHYB levels always appear to be unchanged (Sharma *et al,* 1993; van Tuinen *et al,* 1996; Weller *et al,* 1996). PHYA from the *pcdl* mutant has been partially purified and assembled *in vitro* with phycocyanobilin, an analogue of the phytochrome chromophore, to yield a photoreversible holoprotein (Weller *et al.,* 1996).

2.9 PHYTOCHROME FUNCTIONS

The phytochromes integrate a number of parameters in the light environment and thereby regulate a wide range of light-mediated responses

(Smith 1995; Kendrick and Kronenberg, 1994) by functioning in different mode of actions. These are defined by Low Fluence Response (LFR), and Very Low Fluence Response (VLFR) activated by extremely low light intensities and High Irradiance Response (HIR) activated by constant exposure to relatively high photon fluxes. HIRs are further subdivided into red light- and far-red light-mediated HIR. In contrast to LFRs neither HIRs nor VLFRs are photoreversible. In addition to this series of distinguishing characteristics, each response is distinct in that it displays a clear fluence threshold at which the response saturates.

Mutants lacking one or several of phytochromes have provided useful information concerning the role of individual photoreceptors and their interaction in transducing light signals (Barnes *et ai,* 1997). The availability of phytochrome genes from several plant species also facilitated an alternative approach to analyze photoreceptor function through their expression in transgenic plants. The functions of phyA and phyB have been defined through analysis of responses to constitutive overexpression of phytochrome sequences (Boylan and Quail, 1989, 1991; Kay *et ai,* 1989; Keller *et ai,* 1989; Cherry *et ai.* 1991; Wagner *et ai,* 1991; McCormac *et al.,* 1993). Overexpression of monocotyledonous phyA in the dicotyledonous tobacco and tomato has shown that introduced heterologus phyA is biologically active (Boylan and Quail, 1989; Kay *et ai,* 1989; Keller *et ai,* 1989) and act through normal signal transduction pathways (Nagatani *et ai,* 1991).

Based on the physiological and genetic analysis, the phytochromes have been postulated to have both overlapping and distinct functions (Nagatani *et ai,* 1991, 1993; Somers *et al.,* 1991; Dehesh *et ai,* 1993; Parks and Quail,

1993; Reed *et al,* 1993,1994; Whitelam *et al,* 1993; Wagner and Quail, 1995; Xu *et al.,* 1995, Smith, 1995). PhyA is the primary phytochrome mediating various plant responses to continuous FR light (McCormac *et al.,* 1993; Dehesh *et al.,* 1993; Nagatani *et al.,* 1993; Parks and Quail 1993; Whitelam *et al.,* 1993) and have very limited significance under continous white light, regardless of the fact that PhyA is predominant molecular species in dark-grown tissues (Somer *et al,* 1991). PhyA is responsible for the FR-HIR inhibition of hypocotyl elongation, cotyledon and apical hook opening (Johnson *et al.,* 1994; Koornneef *et al,* 1980; Nagatani *et al,* 1993; Parks and Quail, 1993; Whitelam *et al,* 1993). Phy A also mediates accumulation of anthocyanin in FR light (Kunkel *et al,* 1996) and the so-called FR-preconditioned blocking of greening (Barnes *et al,* 1996a; Runge *et al,* 1996; van Tuinen *et al,* 1995a).

PhyA regulates the induction responses via the VLF mode of phytochrome action such as seed germination (e.g., Botto *et al,* 1996; Parks *et al,* 1996; Mazella *et al,* 1997; Shinomura *et al,* 1996) phototropism (Parks *et al,* 1996; Janoudi *et al,* 1997) and gravitropic orientation of hypocotyl (Hangarter, 1997; Poppe *et al,* 1996; Robson and Smith, 1996). The VLFR mediated by phyA do not require continuous light, and are induced by R, FR, or any wavelength between 300 and 800 nm (Botto *et al,* 1996; Shinomura *et al,* 1996; Mazella *et al,* 1997). The VLF mode of phytochrome action are characterized by a lack of R/FR reversibility and it has been suggested that phyA action during induction reactions is not photoreversible (Furuya and Shcafer, 1996). Apart from VLFR and FR-HIR responses, phyA may also play a role in mediating inductive responses to pulses of R. For instance, Parks *et*

ai, (1996) have shown that, phyA seedlings show no marked enhancement of first positive phototropic curvature.

A major role for phyB in many light-grown seedlings is the perception to R pulses or to continuous R irradiation (e.g Reed *et ai,* 1993, Quail et al, 1995), alteration in R:FR ratio (Smith, 1995) and EODFR. The phyB seems to be the major contributor to the red/far-red reversible low-fluence responses (Furuya and Schafer, 1996; Casal *et al.,* 1998). The phyB also plays a dominant role in the shade-avoidance reaction including the effects of red light and of the R:FR ratio (Smity 1995; Smith and Whitelam 1997; Mc Cormac *et ai,* 1993;Devlin *et al.,*1992 ;Nagatani *et ai,* 1991,1990; Somers *et al.,* 1991). In light-grown seedlings, the LFR is manifested as responses to end-of-day (EOD) light pulses, such as the promotion of elongation growth or flowering by EOD-FR (e.g. Lopez-Juez *et al.,* 1990; Nagatani *et ai,* 1991; McCormac *et ai,* 1993; Parks and Quail 1993; Halliday *et ai,* 1994; Devlin *et ai,* 1996). A further role for solanaceous phytochrome B1 has been revealed in potato where it has been demonstrated that plants transformed with an antisense PHYB lose photoperiodic control of tuberization (Jackson *et ai,* 1996).

Although the responses to continuous R are largely mediated by phy B (Koornneef *et al.,* 1980; McCormac *et ai.* 1993; Parks and Quail 1993) a residual effect can often to observed that suggests the action of other phytochromes (Casal 1995). An *Arabidopsis* phyD mutation was identified as a naturally occurring allele, which encoded no functional phyD protein (Aukerman et al., 1997). The monogenic *phyD* mutant plants had no obvious phenotypic abnormality, whereas plants impaired in both the *PHYB* and *PHYD* genes flowered earlier than the phyB monogenic mutation (Aukerman et al.,

1997; Devlin et al., 1999b). This indicates that, like phyB, phyD inhibits flowering. A genetic screen was carried out to look for mutations that exhibited elongated rosette internodes, resulting in the isolation of the phyE mutation (Devlin et al., 1999a). The *phyE* mutant showed no phenotypic alteration unless it was in the *phyB* mutant background. This indicated the function of phyE is also similar to that of phyB.

2.10 INTERACTIONS BETWEEN PHYTOCHROMES

PhyA and PhyB are capable of mediating some common responses following brief or prolonged R irradiation and share the control of responses but little is known about their interactions in the mutual signaling regulation. Synergistic interactions between phyA in its HIR mode of action and phyB (Casal, 1995) have been reported for hypocotyl growth and cotyledon unfolding in *Arabidopis.* In contrast, phyA in its VLF mode of action may act antagonistically with phyB (Mazzella *et al.,* 1997; Yanovsky *et al.,* 1997). Recent evidences indicate that PhyA acting in VLFR mode is antagonistic to phyB signaling whereas PhyA acting in the HIR mode operates synergistically with phyB signaling in the control of *Lhcbl*2* and hypocotyl growth in *Arabidopsis.* (Cerdan *et al.,* 1999).

The redundant activities of phyA and phyB were revealed when the null mutations, *phyA-201* and *phyB-8-36*, were compared with the double mutant (Reed *et al.,* 1994). Double mutant plants germinated under white, red, or far-red light consistently show longer hypocotyls than do single mutants, suggesting that these two phytochromes partially compensate for each other's activity during hypocotyl elongation. In red light, the double mutant displays enhanced defects in greening and produces a more pronounced apical hook and

smaller cotyledons than either single mutant. The *phyA phyB* double mutant also displays severely impaired induction of chlorophyll a,b binding protein (CAB) transcription by red light in comparison with CAB accumulation in the single mutants. More recently, Devlin *et ai,* (1996) have reported another example of redundancy between phyA and phyB. Mature plants of the *phyA,phyB* double mutant, grown under 10 h light, 14 h dark photoperiods, have reduced stature with only about 30-40% of the biomass of wild type plants. The biomass of individual monogenic phyA or phyB mutants plants is not significantly different from that of wild-type plants (Devlin *et ai,* 1996).

PhyD and PhyE also perform specific regulatory functions in *Arabidopsis* that only partially duplicate those of phyB (Aukerman *et ai,* 1997). Loss of phyD causes alteration of many of the same shade avoidance responses which are affected in the phyB mutant, but comparison of the two null mutants shows that phy B plays a much more prominent role than phyD. Hence, diversification of the PHY gene family, has allowed the evolution of distinct photosensory roles for the photoreceptor subfamilies, with the most divergent genes, exemplified by PHYA and PHYB, having highly divergent functions and the most closely related genes, PHYB and PHYD, having overlapping or even somewhat redundant roles. In addition to redundant actions photoreceptors can also work in antagonistic ways to inhibit responses (Reed et a., 1994; Devlin et al., 1996; Neff and Chory, 1998).

2.11 BLUE/UV-A LIGHT RESPONSES

There are a wide variety of blue-light responses in higher plants. These include the blue-light-induced suppression of hypocotyl elongation, phototropism, anthocyanin induction, expression of specific blue-light

regulated genes, opening of stomata and flowering (Kaufman, 1993). Many such responses show action spectra that are consistent with a flavin chromophore, with peaks of activity in the near-UV (around 350 nm) and blue (450-480 nm) regions of spectrum indicating that these class of responses are mediated by photoreceptors distinct from phytochromes. However, due to the difficulty of obtaining a good *in vitro* assay system for the pigment, there was only recently little progress made towards identification of these receptors (Briggs and Huala, 1999; Lin, 2000).

2.12 BLUE/UV-A LIGHT MUTANTS

Several mutants have been identified that lack either growth inhibition to blue light. The long hypocotyl *{hy-4:* Koornneef *et al,* 1980) and blue light uninhibited (*blu*:Liscum and Hangarter, 1991) mutants of *Arabidopsis* show deficient hypocotyl growth responses to blue light. Although the *blu* mutants were at first identified to be at different loci to *hy4,* however, subsequent analysis showed that they are alleles of *hy4* (Jenkins *et al,* 1995). The ***hy-4*** mutant selectively lacks the inhibition of hypocotyl elongation when grown under B, however, the mutant is normal with respect to far-red light (FR) and red light (R) *hy* responses mediated by phyA and phyB (Koornneef *et al.,* 1980). On the other hand, the cryptochrome *(cry2)* mutant, seedlings have longer hypocotyls than the wild type under relatively low fluence rates of blue light (Lin *et al.,* 1998), which also flowers late in LD (long day) but not in SD (Short day) photoperiods and impaired in photoperiod sensing (Guo *et al.,* 1998). *Cry2* is found to be allelic to a previously isolated photoperiod-insensitive flowering time mutant *fha* (Guo *et al,* 1998; Koornneef *et al,* 1991).

The physiology of phototropism is complex and the relationship of phototropic response to the fluence of unilateral light has been studied in different plant species (Iino, 1990). In most species studied, two types of positive responses are observed, separated by indifferent zone in which very small, no or even negative response is seen. 'First positive' phototropism is characterized by relatively weak response induced by short exposures to relatively high fluence rates. In contrast, the magnitude of 'second positive' phototropism induced is large, and is a direct function of exposure time. The phototropism is further complexed by the fact that after red light pre-irradiation an increase in seedling responsiveness takes place, known as enhancement of phototropism (Janoudi et al., 1990,1991 and 1992). Mutations have been identified that affect first and second positive phototropism in *Arabidopsis.* Liscum and Briggs (1995) isolated non-phototropic mutants (*nph*: for *n*on-*p*hototropic *h*ypocotyl) at four loci (*NPH1-4*)in *Arabidopsis.* Mutants JK224 and 218 isolated by Khurana and Poff (1989) are now found to be alleles of *nph1* and *nph3* (Liscum and Briggs, 1995). Two additional mutants, ZR8 and ZR19, that show normal second positive phototropism, but reduced first positive phototropism (Khurana *et al.,* 1989) were also isolated.

2.13 BLUE /UV-A PHOTORECEPTORS

The use of mutants with known phenotypic defects has provided some information about the nature of blue/UV-A photoreceptors. There are now evidences for more than one distinct BL photoreceptor for blue light induced responses (Liscum *et al,* 1992; Liscum and Briggs, 1995; Ahmad and Cashmore 1996; Short and Briggs 1994; Lazava et al., 1999). For example, studies on blue light mediated hypocotyl growth inhibition and phototropic responses have provided evidence that they are physiologically and genetically

independent (Liscum *et al.*, 1992) and are regulated by different photoreceptors *viz.*, cryptochromes (Ahamad and Cashmore, 1993), and phototropin (Huala *et ai*, 1997) respectively.

2.14 CRYPTOCHROME RECEPTORS - MOELCULAR PROPERTIES

The *HY4* gene, affecting hypocotyl inhibition in BL, has been isolated by T-DNA tagging (Ahmad and Cashmore 1993) which encodes a blue light photoreceptor named cryptochrome I (CRY1; Lin *et ai*, 1995). This photoreceptor has been shown to be responsible for blue light perception by various lines of evidences. The N-terminal two-thirds of CRYl shows 30 % sequence homology to microbial class I DNA photolyase, a kind of flavoprotein. However, the CRYl lacks any detectable photolyase activity, and it also lacks a tryptophan (W227 in the *E.coli* photolyase sequence; Li and Sancar, 1990) residue found in photolyases and believed to be important for binding to pyrimidine dimers (Ahmad and Cashmore, 1993; Lin *et ai*, 1995). Disruption of the region adjacent to the conserved chromophore binding motif found in all class I photolyases and in CRYl(Ahmad and Cashmore, 1993) of *E.coli* photolyase completely inactivates the enzyme. The C-terminal region of CRYl is functionally important as lesions within the corresponding region of the HY4 gene confer a mutant phenotype (Ahmad and Cashmore, 1993; Ahmad *et ai*, 1995).

Secondly, analysis of recombinant CRY1 expressed in and purified from insect and bacterial cells shows that the recombinant protein from insect cells binds FAD noncovalently and in its oxidized state, has an absorption spectrum expected for a blue/UV-A light photorecetor (Lin *et ai*, 1995a, 1995b). In bacterial cells, the recombinant CRYl protein binds a pterin,

methenyltetrahydrofolate (MTHF), in addition to FAD (Malhotra *et al.,* 1995). This pterin chromophore is most likely responsible for the majority of the blue-light absorbing properties of CRY1. Finally, overexpression of CRY1 in transgenic tobacco or *Arabidopsis* plants resulted in plants that **were** hypersensitive to light; the transgenic plants showed an enhanced *hy* response and increased production of anthocyanin (Lin *et al.,* 1995, 1996a). These enhanced responses were observed under B, UV-A, green or white light, but not R or FR. Recently, *CRY1* gene *(TCRY1)* has been identified in tomato that shows 78% identity **and** 88% similarity to *Arabiopsis* CRY1 (Ninu *et al,* **1999).**

A second gene related to CRY1 was isolated from *Arabidopis*, which was named *AT-PHH1* (Hoffman *et al.,* 1996), or *CRY2* (Lin *et al.,* 1996a). The CRY2 is similar to CRY1 but contains a distinct C-terminal sequence (Lin *et al.,* 1996b). The CRY2 gene encodes a protein of 612 amino acids, which is 54% identical with CRY1 within the N-terminal 500 aminoacids. CRY2 does not code for a photolyase (Hoffmann *et al.,* 1996). Another member of cry gene family the *Sinapis alba* gene SA-PHR-1, lacks a C-terminal extension and, like *Arabidopsis* CRY1, its protein product is devoid of DNA photoreactivating activity both *in vitro* and *E.coli* (Batschauer, 1993; Malhotra *et al.,* 1995). Further, CRY genes have been isolated from organism other than higher plants, including *Chlamydomonas* (Small *et al,* 1995).

2.15 PHOTOTROPIN RECEPTOR- MOLECULAR PROPERTIES

The *NPH1* gene of *Arabidopsis,* affecting phototropic responses, encodes blue light photoreceptor known as phototropin that is essential for phototropism and is a plasma membrane-associated phosphoprotein (Lin *et* ***at,***

1998). The phototropin is an apoprotein of 996 aminoacids (112 kDa) and the coding region consists of 20 exons extending for 5.4 kb. The deduced amino acid sequence suggests that the C-terminal region of the gene encodes a serine/threonine protein kinase. The N-terminal region of the gene has two repeated domains, LOV1 and LOV2, that share similarity with diverse proteins of archaea, eubacteria and eukaryotes that detect changes in redox status as affected by light, oxygen, or voltage (hence LOV) (Huala *et ai,* 1997). This LOV domains binds flavin chromophore (Christie et al.1999).

The putative phototropin photoreceptor has been shown to be blue light photoreceptor for phototropism by the following evidences. In the plant, it becomes phosphorylated on exposure to blue light; this reaction was detected as biochemical step necessary for blue light signal transduction before identifying it as a receptor (Gallagher *et ai,* 1988; Reymond *et ai,* 1992). The spatial and temporal, fluence and dark-recovery kinetics of the phosphorylation response correlate well with phototropic response which led to the conclusion that the phosphorylation event was likely to be a part of the phototropism signal transduction pathway (Short and Briggs, 1990, 1994). In addition, a gradient asymmetric distribution of phosphorylation of the protein within the coleoptile at the irradiated versus the shaded side of oat coleoptiles has been demonstrated (Salomon *et ai,* 1997a, 1997b).

Secondly, the detailed biochemical and genetic analysis of *Arabidopsis* mutants lacking phototropic responses further supported this correlation. The *hy-4,* coding for cryptochrome blue light receptor showed normal phototropism indicating that it is not involved in phototropism. On the other hand, Reymond *et al.,* (1992) showed that *Arabidopsis* strain JK224, which has a threshold

fluence for first positive curvature approximately 100-fold greater than wild-type, was deficient for the light-induced phosphorylation. Furthermore, the *nph1* mutant allelic to JK224 mutant with the concomitant loss of the 120 kD protein in all its null alleles and of any blue light-dependent phosphorylation, gave rise to the hypothesis that phototropism may be regulated by a photoreceptor that becomes phosphorylated upon irradiation with blue light (Liscum and Briggs, 1995). Finally, by expressing the NPH1 gene in insect cells found that the protein produced had exactly the same light sensitivity as the protein from the plant (Huala et al., 1997). Hence, it was proposed that NPH1 was the photoreceptor for the phototropic response.

2.16 BLUE/UV-A PHOTORECEPTORS FUNCTIONS

In *Arabidopsis,* CRY1 has been implicated in developmental responses like blue light-induced inhibition of hypocotyl elongation, stem growth and internode elongation, leaf and cotyledon expansion, B-dependent gene expression, and anthocyanin accumulation (Ahmad and Cashmore 1993; Ahmad and Cashmore, 1996; Fuglevand *et al,* 1996; Koorneef *et al,* 1980; Lin *et al.,*1995b; Lin *et al.,* 1995a; Ninu *et al,* 1999).

Mutant studies and transgenic plants have enabled identification of the function of CRY2. The late flowering *Arabidopsis* mutant *fha-1* (Koornneef *et al,* 1991) is defective in the CRY2 gene (Guo *et al,* 1998), indicating that CRY2 is a blue light photoreceptor which is involved in measuring the day-length. Transgenic plants overexpressing CRY2 were hypersensitive to blue light and developed short hypocotyls under blue light or white light (Lin *et al,* 1998) and *cry2* mutant seedlings had longer hypocotyls that the wild type under relatively low fluence rates of blue light (Lin *et al,* 1998), indicating that the

function of *cry2* in hypocotyl inhibition was limited to low intensity light. This result was interpreted as being the consequence of the blue-light-induced degradation of CRY2 protein in high intensities of blue light (Lin *et al,* 1998). Specifically, overexpression of CRY2 resulted in substantial increase in the sensitivity of cotyledon expansion to blue light (Lin and Cashmore, 1996).

Extensive biochemical, physiological and genetic work done has shown that NPH1 is the only photoreceptor for both first and second positive phototropism (Huala *et al,* 1997; La 1999). In addition, the CRY1 in combination with CRY2 are implicated in first positive phototropism (Ahmad *et al.,* 1998) but recent analysis showed that phototropin alone is the main photoreceptor and CRY1 and CRY2 only modulate the response downstream of the receptor (Lasceve et al., 1999).

CO-ACTION OF PHOTORECEPTORS

2.17 CO-ACTION OF PHYTOCHROMES AND BL/UV-A PHOTORECEPTORS

Though phytochromes and blue/UV-A photoreceptors employ distinct signal transduction pathways (Kunkel *et al.,* 1996; Yanovsky *et al.,* 1997), these receptors display an intense interactions (Casal, 2000). Numerous physiological experiments on inhibition of hypocotyl growth and photoregulation of anthocyanin have documented cases of synergy or interdependent co-action between phytochrome and the BL photoreceptor(s) (Mohr, 1994). In independent co-action both photoreceptors can elicit the same response, interdependent co-action can be of several possibilities(Mohr, 1994). Nevertheless, while the mode of co-action and the relative importance of different photoreceptors is still unclear; some species appear to respond only to phytochrome and blue/UV-A photoreceptors (Gaba and Black, 1979).

The study of co-action is inevitably further complicated by the fact that B/UV-A is also absorbed by phytochrome leading to Pfr production **and** phytochrome cycling. It can absorb blue light both *in vitro* and *in vivo* (Butler *et ai,* 1964; Jabben *et ai,* 1982). However, the phytochromes are generally not regarded as blue/UV-A photoreceptors because they operate maximally in red and far-red regions of visible light. Hence, it has been hypothesized that the blue light receptor function is independent of phytochromes (Cosgrove, 1981; Gaba and Black, 1987) or the blue light receptor is completely dependent on the presence of active phytochrome and only modulates the sensitivity of phytochrome signal transduction cascade (Oelmuller and Mohr, 1985). Mohr (1994) proposed a model of co-action between BAJV-A photoreceptors and the phytochrome photoreceptor system. The Pfr is considered as the effector while the B/UV-A effect is considered as amplifying responsiveness toward Pfr. The general observation seems to be that the responsitivity towards Pfr can be strongly increased - even induced - by a pre-irradiation, which is absorbed by cryptochrome. Recently, the stimulation of responsiveness to Pfr was not only detected after B but also after FR pre-irradiation (Beggs *et al,* 1981; Casal, 1995).

As a consequence crucial question was addressed as to whether blue light responses are mediated by phytochromes and/or blue light photoreceptors. The genetic evidences obtained from phytochrome deficient mutants indicate that phytochrome can also act as blue light receptor in different responses. Under continuous BL, phytochrome deficient mutants show virtually wild-type responses suggesting that phyA and BL photoreceptors act independently in an additive manner and contributes to the blue light induced hypocotyl growth inhibition response (Koornneef *et ai,* 1980; Young et al. 1992; Liscum and

Hangarter, 1994). By using phyA, phyB, and phyAphyB double mutants of *Arabidopsis*, it has been shown that PHYA is the most sensitive blue light receptor for the induction of seed germination (Shinomura *et al.*, 1996) or LHCB gene expression in VLF. Moreover, PHYB and an additional phytochrome of unknown identity contribute to a LF blue light induction of LHCB which shows far-red light reversibility (Hamazato *et al.*, 1997). Similarly, by using double mutants *phyA,phyB* it has been shown that the presence of either phyA or phyB is required for first positive phototropism and time threshold of second positive phototropism (Janoudi *et al.*, 1997; Hangarter, 1997). Furthermore, it has been demonstrated that white and blue light induced accumulation of anthocyanin requires the presence of at least one of the phytochromes: either phyA or phyB (Kunkel *et al.*, 1996; Ahmad and Cashmore, 1997).

Evidence for the blue/UV-A receptor acting through phytochrome is further supported by studies in other plant species. The *au* mutant of tomato deficient in all phytochromes retains some responses to BL and lacks others (Oelmuller and Kendrick 1991; Oelmuller *et al*, 1989; Casal 1994). Levels for several light regulated nucleus encoded transcripts in wild-type tomato show a blue-light-induced increase beyond the levels produced by red light alone. However, neither red nor blue light leads to accumulation of these transcripts in the *au* mutant, showing that blue light is ineffective in the absence of phytochorme in this system (Oelmuller et al., 1989). In the case of the *lh* mutant of cucumber, deficient in phyB (Lopez-Juez *et al.*, 1992) light-grown plants do not respond to BL (Ballare *et al.*, 199;1 Lopez-Juez *et al.*. 1992). In contrast, the overexpression of oat phyA impairs the response mediated by BL receptor(s) (Casal and Sanchez, 1994).

Another example of interacting photosensory systems is evidenced by the ability of phytochrome to enhance blue-light mediated phototropism (Steinitz *et al.,* 1985). It has been found that the red-light preirradiated seedlings usually exhibit an enhanced phototropic curvature in response to subsequent stimulation by unilateral blue light (Chon and Briggs, 1966; Janoudi and Poff, 1991, 1992). Analysis of the various phytochrome deficient mutants indicates that phy A is primary photoreceptor responsible for the enhancement response by low fluences of red light (Parks *et al.*, 1996). Moreover, there are indications for a response-specificity in the action of phy A and phyB (Parks *et al.,* 1996). By studying phy A and phyB mutants and transgenic lines overexpressing these phytochrome species, Janoudi *et al.,* (1997a, b) have indicated that the involvement of phy A is essential in the very-low to low-fluence range for enhancement and either phyA or phyB is required for the high-fluence enhancement by red light. However, the nature of the interaction between phytochromes and phototropic response is not understood.

Different species of phytochromes and cryptochromes are known to interact synergistically or antagonistically following a brief or prolonged B irradiation and share the control of response. Though CRY1 interacts with PHYB, it does not interact synergistically with PHYA (Casal and Boccalandro, 1995). Moreover, measurements of blue-light induced shrinkage of protoplasts indicate that PHYB was mainly responsible for the phytochrome dependence of this CRY1 mediated response and not PHYA (Wang and Iino, 1997). In contrast, Ahmad and Cashmore (1997) observed reduced responses to blue light compared with darkness in *phyA,phyB* mutant of *Arabidopsis* and proposed that PHYA or PHYB is necessary for CRY1 activity. Recent investigations indicate an interaction of CRY1 and PHYA *in vitro* (Ahmad *et*

ai, 1998). Further, the physiological and genetic studies demonstrate that CRY2 suppresses or antagonistic to the function of the PHYB-dependent inhibition of floral initiation (Guo *et ai,* 1998; Mockler *et ai,* 1999).

LIGHT SIGNAL TRANSDUCTION ELEMENTS

2.18 PHYTOCHROME SIGNAL TRANSDUCTION - BIOCHEMICAL ELEMENTS

Though considerable progress has been made in identifying the photoreceptors that mediate the effects of light and the *cis*-elements and transcription factors that are involved in the photoregulation of specific genes, however, the signal transduction processes that couple photoreception to transcription remains fragmentary. Several components of phytochrome signal transduction pathways have been identified by biochemical methods (Millar *et ai,* 1994). There is evidence that G protein activation is an early event (Neuhaus *et ai,* 1993; Romero and Lam, 1993), and transient increases in cytosolic calcium ions have been reported (Roux *et ai,* 1986; Chae *et ai,* 1990; Shacklock *et al.,* 1992; Volotovski, 1998).

Using a combination of microinjection of pharmacological agonists and antogonists in *au* mutant of tomato, the phyA signaling has been shown to involve the activation of one or more heterotrimeric G proteins and the subsequent participation of three different pathways dependent upon calcium and/or cGMP. The calcium-dependent pathway regulates the expression of genes such as CAB and is able to direct partial chloroplast development. The cGMP-dependent pathway regulates expression of CHS and production of anthocyanin pigments. A third calcium/cGMP-dependent pathway is required for FNR gene expression and full chloroplast maturation (Bowler *et ai,* 1994a). These pathways cross-regulate each other by reciprocal control (Bowler *et ai,*

1994b). For example, high levels of cGMP can negatively regulate the two calcium-dependent pathways, and high levels of calcium or calcium-activated calmodulin can negatively regulate the cGMP pathway that controls CHS expression.

2.19 PHYTOCHROME SIGNAL TRANSDUCTION - GENETIC ELEMENTS

Wagner *et al.,* (1997) recently proposed the hypothesis of specific pathways of signal transduction downstream from phyA and phyB. This possibility is supported by the observations that loci such as *fhy1, fhy3* (Whitelam *et ai,* 1993), *vlf1* and *vlf2* (Yanovsky *et ai,* 1997), *fin 2* (Soh *et ai,* 1998) and the *spal* have been genetically identified in the PhyA signaling pathway that affect phyA- mediated responses but not phyB- mediated responses. The mutants denoted *fhyl* and *fhy3,* are not linked to the PHYA gene. They resemble phyA-deficient mutants despite possessing normal levels of spectrally active PHYA protein and normal levels of PHYA mRNA (Whitelam *et ai,* 1993). The *fhyl* mutant is blocked, however, in only a subset of phyA-mediated responses at the physiological level, as phy-A dependent effects on germination are normal in *fhy1* mutants (Johnson *et ai,* 1994), suggesting that *fhy-1* defines a branchpoint in phyA signal transduction pathways. Indeed, this has recently been demonstrated at the level of gene expression; *fhy1* is deficient in phyA-regulation of only a subset of genes, such as CHS, whereas the regulation of genes such as CAB and nitrate reductase (NR) is relatively intact (Barnes *et ai,* 1996). By contrast, the *pef2, pef3,* (Ahmad and Cashmore, 1996) and *red1* (Wagner *et al.,* 1997) mutants have reduced de-etiolation only in R, indicating that these loci may be specific to phyB - but not phyA-mediated signal transduction and act as positive regulators in the signaling pathway.

The mutant *spal* also specifically affect only responses to FR and, hence, phyA signaling (Hoecker *et ai,* 1998). Compared to *fliyl* and *fliy3* mutants which show impairment in phyA -regulated response, the *spa1* mutant shows amplified phyA signaling and is thought to be mutated at a locus encoding a negatively acting signaling component. Very recently, it has been shown that the SPA1 encodes a nuclear-localized 115 kDa protein with WD-repeats as found in COP1 (Hoecker *et ai,* 1999). Because of their resistance to far-red-induced death and their inability to respond to PHYA-mediated gene expression, the *pat* mutants are also related to PHYA signaling (Cordelia Bolle *et al.,* **1999).**

Although phyA and phyB activities occur under different light conditions, the end-point responses (e.g., hypocotyl growth, cotyledon unfolding, flowering etc.,) controlled by phyA and phyB are largely same. The phototransduction pathways of phyA and phyB obviously converge at some point. The relative position of the point of convergence is not known but it has been proposed that phyA and phyB share the same reaction partner (Wagner *et al.,* 1996a, 1996b; Ahmad and Cashmore, 1996). The mutants such as the *pefl* mutant (Ahmad and Cahsmore, 1996) shows reduced R- and FR- mediated responses, whereas the *psi 1* (for phytochrome signaling) mutant (Genoud *et ai,* 1998) shows enhanced R-and FR- mediated responses and thought to encode negatively acting component, suggesting disruption in both phyA and phyB signaling.

2.20 PHYTOCHROME SIGNAL TRANSDUCTION - INTERACTING PARTNERS

Several phytochrome interaction partners have been isolated by yeast two-hybrid system. A new type of basic helix-loop-helix type protein (pif3) has

been identified as possible signal-receiving protein and constitutively localized in the nuclei with similarities to b-HLH transcription factors (Ni et al., 1999). Pif 3 contains a single PAS-like domain in its N-terminal region, and specifically interacts with the C-terminal domains of both phyA and phyB. Transgenic plants overexpressing the *PIF3* gene in antisense orientation show reduced responsiveness to red and far-red treatments. Furthermore, the interaction of PIF3 with the mutants derivatives of the C-terminal domains of phyA and phyB, was also severely compromised *in vitro.* These data suggest that PIF3 is indeed required for phyA and phyB signaling and its interaction with phyA and phyB is probably mediated by the PAS-like domains present in these proteins. Recently it has been shown that the photoactive phytochrome B binds PIF3 *in vitro* only upon light-induced conversion to its active form, and that photoconversion back to its inactive form causes dissociation from PIF3 (Ni *et al.,* 1999) providing a potential mechanism for direct photoregulation of gene expression.

Moreover, recently Martinez Garcia et al., (2000) have shown that phytochrome B binds reversibly to G-box-bound PIF3 specifically upon light-triggered conversion of the photoreceptor to its biologically active conformer, suggesting that phytochromes may directly regulate the transcriptional machinery of specific genes by physically complexing with promoter bound transcriptional regulators. Using a similar approach, Fankhauser et al., (1999) have isolated a protein designated phytochrome kinase substrate 1(PKS1) which binds to both PHYA and PHY C-terminal domains. Choi et al., (1999) have also isolated nucleoside diphosphate kinase 2 as phyA-interactor which is involved in phytochrome signaling. These studies have thus identified potential immediate signaling partners for phytochrome A and B.

2.21 BLUE/UV-A SIGNAL TRANSDUCTION- BIOCHEMICAL ELEMENTS

Warpeha *et al.*, (1991) have identified a **blue-light activated** heterotrimeric GTP- binding regulatory protein associated with the plasma membrane of pea apical buds. The threshold fluence for blue-light excitation of the G-protein resembles that for the blue-light induced transcription of the *Cab* gene family in pea. Plasma membranes derived from the apical buds of peas exhibit GTPase activity and GTP-y-S binding when irradiated with blue light but not when irradiated with red light. The a-subunit was identified as **a** 40-kD polypeptide by several means, including cross-reactivity with polyclonal antibodies directed against transducin, blue-light-specific binding of a photoaffinity-labeling GTP analog, blue-light specific ADP-ribosylation by chloera toxin, and blue-light-specific inhibition of ADP-ribosylation by pertussis toxin. The receptor driving the G-protein activity is likely to be a flavoprotein (Warphera *et ai,* 1992). Compounds such as phenylacetate and potassium iodide, inhibiting transfer of excitation energy from flavins to nearby proteins, inhibit the ability of blue light to activate the G-protein.

As for the blue light and UV receptors responsible for anthocyanin accumulation in *Arabidopsis*, the effects of pharmacological agents indicated that an increase in cytoplasmic Ca2+ is somehow involved in, although not sufficient to cause, the light-induced increase in CHS mRNA in suspension-cultured cells (Christie and Jenkins, 1996). Also, the effects of kinase **and** phosphatase inhibitors indicate **a** role for phosphorylation in the signal cascade (Christie and Jenkins, 1996).

2.22 BLUE/UV-A SIGNAL TRANSDUCTION - BIOPHYSICAL CHANGES

The blue light dependent inhibition hypocotyl elongation initiates within seconds of blue-light exposure, and results from a change in the cell wall's ability to relax and expand (Cosgrove, 1988). The most immediate effect of blue-light irradiation is a transient hyperpolarization of the plasma membrane of cucumber hypocotyl cells (Spalding and Cosgrove, 1992). The hyperpolarization, as large as 100 mV, precedes the cessation of stem elongation and is rectified within 2 to 3 min. The inhibitory effects of vanadate and KCN strongly suggest that depolarization is due to plasma membrane H+-ATPase. Repolarization appears to involve calcium channel. Membrane depolarization correlates with the suppression even both temporally and with respect to the threshold of the response. Furthermore, the CRY1 could mediate the activation of anion channels which may lead to plasma membrane depolarization was shown to be involved in blue-light-dependent growth inhibition (Cho and Spalding, 1996; Parks *et al.,* 1998).

2.23 BLUE/UV-A SIGNAL TRANSDUCTION - GENETIC ELEMENTS

Genetic studies have defined the elements acting downstream of phototropin in phototropism by isolating mutants *nph-3* and *nph-4* (Liscum and Briggs, 1995). Further the studies have shown that *nph-4* acts downstream of *nph-3* and acts as a conditional modulator of auxin-dependent growth responses in *Arabidopsis* (Stowe-Evans et al., 1998). Recently, both *NPH3* and *NPH4* genes were cloned by positional cloning. The *NPH3* encodes a protein of unknown function that interacts *in vitro* with phototropin (Motchoulski and Liscum, 1999). The *NPH4* gene product shows high homology to auxin-regulated transcriptional regulators (Harper et al., 2000).

2.24 TRANSLOCATION OF RECEPTORS TO NUCLEUS

The phytochromes and cryptochromes are known to translocate in a light dependent fashion into the nucleus. R-dependent import of PHYB-GUS fusion protein into the nucleus (Sakamoto and Nagatani, 1996). Interestingly, the nuclear import of PHYA is dependent of FR and PHYB upon R treatment. The CRYl photoreceptor was found to be a soluble protein expressed at similar levels in dark and light grown *Arabidopsis* seedlings (Lin *et al,* 1996a, 1996b), but was also found to be enriched in the membrane fraction (Ahmad *et al,* 1998a). Very recently it was shown that a fusion protein consisting of CRYl and GFP localizes to the nucleus, indicating that CRYl is a nuclear protein (Cashmore *et al.,* 1999). Arabidopis CRY2 is also localized to the nucleus (Kleiner *et al,* 1999; Guo *et al.,* 1999). The CRY2 contains a putative nuclear localization signal (NLS) within its C-terminal region (Kleiner *et al,* 1999).

2.25 POSITIVE REGULATORS OF LIGHT SIGNAL TRANSDUCTION

Although it appears that the blue-light and phytochrome photosensory systems act in an independent and additive manner, they probably have some common elements in their signal transduction systems. This hypothesis is supported by the *hy5* mutant of *Arabidopsis,* which exhibits reduced hypocotyl growth inhibition to red, far-red and blue light, and produces less anthocyanins in the light than do wild type plants (Koornneef *et al.* 1980). The ***hy-5*** is also unlinked to photoreceptor mutations and possesses normal amounts of spectrally active phytochrome (Koornneef *et al,* 1980; Chory *et al,* 1989a; Parks *et al,* 1989; Nagatani *et al,* 1991; Somers *et al,* 1991). Furthermore, the *hy 5* mutant appears to be at least partially epistatic to the phytochrome-deficient mutants (Koornneef *et al,* 1980) and *hy4* (Chory 1992) suggesting that the HY5 gene product is a common downstream element in the red/far-red-

and blue-light response pathways (Chory 1992, 1993). Recently, the HY5 has been identified as a protein with homology to b-ZIP transcription factors.

2.26 CONSTITUTIVE AND EXAGERATED PHOTOMORPHOGENEIC MUTANTS

Putative constitutive-response mutants have been screened with light-grown morphologies when grown in the dark. Such mutations have been isolated from three plant species: the'de-etiolated'(*det*), 'constitutively photomorphogenic'(*cop*) and '*fusca* '(*fus*) mutants (Chory *et al.*, 1989; 1991; Deng *et al.*, 1991; Misera *et al.*, (1994)) of *Arabidopsis*, *Pisum sativum* L, (Frances *et al*, 1992; Kwok *et al.*, 1996), and tobacco (Trass *et al.*, 1995) and all mutations identified to date are recessive. Their dark-grown seedlings have lost some of the characteristics of the dark developmental pathway and exhibit aspects of photomorphogenic development. In addition, two suppressor mutants *shy* (suppressor for *hy2*) at two loci (*shy 1-2)* of long hypocotyl mutant (*hy-2*) showing photomorophogenic phenotype in dark are identified in *Arabidopsis* which are dominant. They are partially constitutive and exhibit phenotypic traits in addition to those due to loss of phytochrome activity and involved farther downstream in signal transduction.

Genetic analysis showed that some of *det/cop* mutants of *Arabidopsis* are allelic to some *fus* mutants (Castle and Meinke, 1994; Pepper *et al.*, 1994). The determination of hierarchy of *det/cop/fus* and the photoreceptor mutants have shown that *det/cop/fus* mutants are epistatic to the latter and act downstream of both the blue-light and phytochrome response systems (Chory, 1992; Ang and Deng, 1994; Misera *et al.*, 1994). Because all of the mutations at the pleotropic COP/DET/FUS loci are recessive and cause photomorphogenic development in darkness, the proteins encoded by these loci

have been postulated to act as repressors of photomorphogenesis in dark. Light signals absorbed by the various photoreceptors are thought to reverse the repressive activities of the COP/DET/FUS proteins and allow photomorphogenetic development to proceed.

The tomato *high pigment (hp-1)* mutant shows exaggerated phytochrome responses and is dwarfed and dark green (Peters *et ai,* 1989, 1992). This phenotype is identical to that of transgenic tomato plants overexpressing phytochromes A and B (Boylan and Quail, 1989; Wagner *et ai,* 1991). However, the *hp-1* mutant does not accumulate higher levels of phytochrome, nor is it defective in the degradation of phytochrome (Peters *et al.,* 1992). The double mutant analysis of *hp-1* with PhyA- and PhyB-deficient tomato mutants has demonstrated that the *hp-1* mutation can amplify responses mediated by both phytochromes (Peters *et al,* 1992; Kerckhoffs *et ai,* 1997b). These results led to the hypothesis that the *HP* gene product is involved in an amplification step in phytochrome signaling and may act as negative regulator.

2.27 NEGATIVE REGULATORS OF LIGHT SIGNAL TRANSDUCTION

The DET1 gene was isolated by positional cloning, and it encodes a hydrophilic protein (Pepper *et ai,* 1994). Recently, a tomato homolog of the *Arabidopsis* DET1 has also been found by cloning *HP-2* gene (Mustilli *et ai,* 1999).The COPl gene encodes a protein with a novel combination of distinct domains: an N-terminal zinc-binding RING-finger, a putative coiled coil, and a domain at the C-terminal with multiple WD-40 repeats homologus to the b subunits of heterotrimeric G-proteins (Deng *et ai,* 1992; Lovering *et al.,* 1993; Mc Nellis *et ai,* 1994a). The N-terminal portion of COPl, which alone is retained in the mild *cop* 1-4 alleles, is sufficient to perform a basal set of

functions that prevent seedling lethality, but the C-terminus is required for the repression of light-inducible genes (Mc Nellis *et al.,* 1994a). The FUS6 gene isolated by T-DNA tagging encodes a novel polypeptide with no homology to known metabolic or regulatory proteins and the identification of its homolog in rice suggests *that fusca* genes may be conserved throughout the angiosperms (Castle and Meinke, 1994). COP9 was cloned from a T-DNA tagged mutant allele, and shown to encode a small hydrophilic protein with a molecular mass of 22.5 kDa (Wei et al. 1994b). However, gel filtration studies indicated that COP9 functions *in vivo* in a high-molecular weigh protein complex of 560kDa (Wei *et al,* 1994b).

Based on biochemical analysis of DET1, COP1, COP9 and FUS6 fall into two subgroups. COP9 and FUS6 are representative of one group, because they copurify as subunits of a 500-kD nuclear-localized protein complex, which may also include COP8 (Chamovitz *et al.,* 1996; Staub *et al,* 1996). The COP9 complex is localized to the nucleus and binds heparin (Chamovitz *et al.,* 1996); however, the biochemical function for this complex is not known. Indirect evidence for the function of the COP9 complex has been provided in animal systems in which proteins highly similar to COP9 complex components have been identified (Chamovitz and Deng, 1995; Spain *et al.,* 1996).

Ecotopic expression of the human FUS6 ortholog, GPS1, inhibits mitogen-activated protein kinase (MAPK) pathways in both yeast and mammalian cells (Spain *et al.,* 1996). The recently reported mammalian COP9 complex contains JAB1, a c-JUN-activating binding protein (Seger *et al.,* 1998; Wei *et al.*. 1998). The human COP9 complex (also termed the signalosome) appears to phosphorylate c and D-Jun *in vitro* (Seger *et al.,* 1998), suggesting that the COP9 complex may be directly involved in MAPK pathways. Recent

reports have highlighted the similarities among the COP9 complex, eIF3 complex, and the 19S regulatory component of the proteasome (Glickman *et ai,* 1998; Wei *et ai,* 1998). All three complexes are multisubunit and similar in size, and subunits of all three complexes share a similar motif, termed the PCI (for proteasome-COP9 complex initiation; Hofmann and Bucher, 1998) or PINT (for proteasome-Int6-Nip1-Trip15; Aravind and Ponting, 1998) domain.

The second subgroup of COP/DET/FUS proteins includes COP1 and DET1. Both COP1 and DET1 were identified to be nuclear regulators (Pepper *et ai,* 1994; von Arnim and Deng, 1994). Chimeric proteins of both DET1 and COP1 fused to bGUS reporter proteins were shown to be nuclear localized and act in the nucleus to suppress photomorphogenic development (Pepper *et al.* 1994; von Arnim and Deng, **1994).** Moreover, light modulation of COP1 activity involves a light-dependent nucleocytoplasmic partitioning of COP1 in hypocotyl cells of seedlings (von Arnim and Deng, 1994). The protein localizes to the nucleus in the dark and to the cytoplasm in the light. The relocalization of the COP1-GUS protein to the nucleus in the dark was blocked in several *cop* mutants and in the *det* mutant, implying that the products of these genes are involved in the dark-induced nuclear targeting of COP1. Furthermore, in a *cop* enhancer/suppressor screen, mutants at the *hy5* locus were recovered; the COP1 and HY5 proteins were subsequently shown to interact in a yeast two-hybrid experiment (Ang et al., 1998).

COORDINATION OF LIGHT SIGNALLING

2.28 INTERACTION OF LIGHT SIGNALS WITH ENDOGENOUS FACTORS

Physiological and molecular studies have begun to yield information about the interaction between light cues and their modulation by endogenous signals. The mutations in less pleiotropic photomorphogenic developmental phenotypes

in darkness such as *cbb1* (Kauschmann *et ai,* 1996); *cpd/cbbi* (Kauschmann *et al.,* 1996; Szekeres *et ai,* 1996); *dwfl,* allelic to *dim/cbb1* (Kauschmann *et ai,* 1996; Takahashi *et ai,* 1995); *det2* (Chory *et ai,* 1991); *cop2/ampa/pt-l, hlsl/cop3* (Chaudhury *et ai,* 1993; Hou *et ai,* 1993; Lehmannn *et ai.* 1996); and *cop4* (Hou *et ai,* 1993; suppressor of *hy2* or *shy* (Kim *et ai,* 1996) suggest that in addition to photoregulated seedling development, the genes may play a role in other aspects of plant development. Physiological and developmental studies by Castle and Meinke (1994) showed that the *fus6(copl 1)* mutation has pleiotropic effects, interfering with normal responses to sugars, hormones, and developmental signals, in addition to light. Indeed, severe mutant alleles of several of these genes (e.g. *copl* and *detl*) are lethal. Further, the *detl*, *copl*, and *cop9* mutations cause inappropriate expression of several genes (Mayer *et ai,* 1996).

Many of the developmental processes that occur as result of light signals are dependent, at least in part, on the action of phytohormones. The phenotypic defects in three of the less pleotropic genes are rescued by treatment with brassinosteriods, suggesting that this class of plant growth regulators is required for efficient hypocotyl cell elongation during the etiolation response (*det2*; Li *et ai.* 1996); *cpd/cbb3* and *dwfl/dim/cbb1* {*Kauschmann et ai,* 1996; Szekeres *et ai,* 1996). The mutants known as *det2* (Chory *et ai,* 1991) and *diminuto* (Takahashi *et ai,* 1995) are shown to be deficient in the biosynthesis of brassinosteriod plant hormones and show partial de-etiolation in darkness suggesting that brassinosteroid hormones play an important role in coordinating the etiolation response (Li *et ai,* 1996; Szekeres *et ai.* 1996). How exactly the brassinosteroid-mediated pathway and the COP/DET/FUS pathway are integrated with each other remains to be shown, but partial rescue

of severe *cop1* mutants by brassinosteroid treatment has suggested that the two pathways are separable (Szekers *et ai,* 1996).

In some other cases, light has been shown to alter the levels of IAA (Bandurski *et ai,* 1977; Jones *et ai,* 1991; Behringer and Davies, 1992), GAs (Ross *et ai,* 1992; Foster and Morgan, 1995), ABA (Kraepiel *et ai,* 1994; Toyomasu *et ai,* 1994); Weatherwax *et ai,* 1996), cytokinins (Qumuruddin and Tillberg, 1989; Kraepiel *et ai,* 1995), and ethylene (Kathiresan *et ai,* 1996). Behringer and Davies (1992) proposed that phytochrome regulation of stem elongation is partly the result of changes in IAA levels. Phytochrome-deficient mutants of *Arabidopsis* require GAs to express the elongated phenotype of these plants (Peng and Harberd, 1997).

On the other hand, carbohydrates have been found to alter responsiveness to light, particularly with respect to specific gene expression (Tsukaya *et ai,* 1991; Cheng *et ai,* 1992; Harter *et ai,* 1993; Dijkwel *et ai.* 1996, 1997). Recent data have shown that metabolizable sugars can overcome the phyA-specific repression of protochlorophyllide oxidoreducatases (Barnes *et ai,* 1996), that Suc represses light-inducible plastocyanin production (Dijkwel *et ai,* 1996), and that Suc can specifically affect seedling growth in FR light (Whitelam *et ai,* 1993; Dijkwel *et ai,* 1997). Furthermore, in *Arabidopsis* overexpression of phyB inhibits phyA function in the presence of sucrose (Short, 1999). However, it is unclear how carbohydrates and phytochrome signaling mechanisms interact to regulate these functions.

CHAPTER 3

MATERIALS AND METHODS

3.1 PLANT MATERIAL, GROWTH CONDITIONS AND SEED COLLECTION

Three varieties of *Lycopersicon esculentum* .L were used: cv. Ailsa craig (AC) or Money maker (MM) or GT breeding line for the different physiological studies and isolation of non-phototropic lines (Ailsa craig). Plants for seed production were raised in a culture shed. Seeds of each genotype were germinated in soilrite mixture and transplanted at cotyledon stage into pots containing soil. Plants were grown in a net house under ambient light conditions with day/night temperatures approximating 30°C and 25°C respectively. Seeds were isolated from mature fruits and fermented for 24 hrs to remove the remnants of the mucilagenous locular tissue. Thereafter the seeds were rinsed with tap water, dried at room temperature and stored in boxes in a refrigerator at 5 °C until use.

3.2 MONOGENIC MUTANT LINES

The following mutant lines were obtained from Prof. Marteen Koornneef, of the Department of Genetics, Wageningen Agricultural University, The Netherlands and Prof. Kendrick of the Laboratory for photoperception and signal transduction, RIK.EN, Japan. The pure lines of mutants were maintained in the Department of Plant sciences at the University of Hyderabad, Hyderabad. The genotypes used are listed in Table 3.1 along with information on their photophysiology and genetic background. The phenotypes of the mutants and their respective wild type seedlings in the same background are also shown in Figs. 3.1 and 3.2.

***au(aurea)*:** The isolation of the long-hypocotyl mutant after mutagenesis of the wild type tomato has been described by Koornneef et al., (1985). It has been shown that the mutant is deficient in light-labile, type 1 phytochrome

Table 3.1 Summary of the genotypes used in this study. Mutants are homozygous recessive for the allele indicated and are isogenic to their respective wild type control. Double mutants related to *tri,fri* and *fri,hp-1* are in the mixed background of respective wild type controls.

GENOTYPE	PHENOTYPE	PHOTOPHYSIOLOGY	CULTIVAR
WT	normal	normal	AC or MM or GT
au	elongated hypocotyl, pale green leaves	insensitive to red light	AC
fii	elongated hypocotyl	insensitive to far-red light	MM
tri	elongated hypocotyl	insensitive to red light	GT
hp-1	short hpocotyl, dark green leaves	hypersensitive to light	AC
tri,fri	elongated hypocotyl	insensitive to light	GT,MM
au.hp-1	elongated but stunted hypocotyl	insensitive to light	AC
fri,hp-1	hypocotyl length normal	--	MM,AC

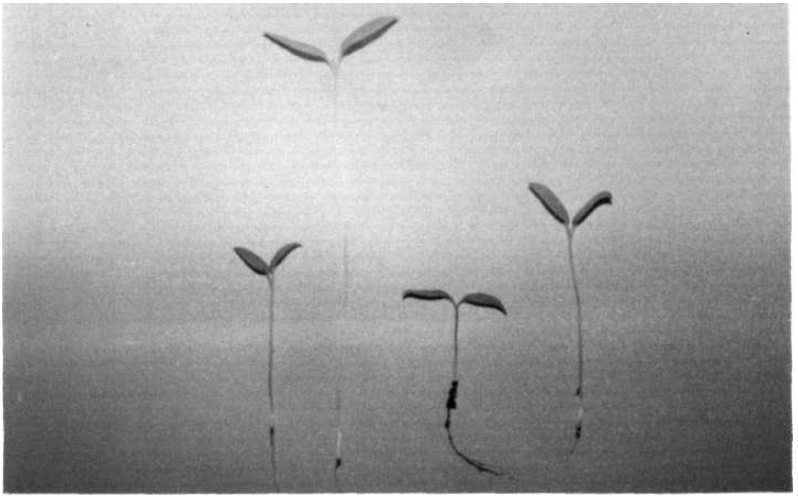

Figure 3.1 Phenotype of 7-day old light-grown mutants in comparison with wild-type seedlings. From left to right wild-type (AC); *au* mutant; *hp-1* mutant; *au,hp-1* double mutant.

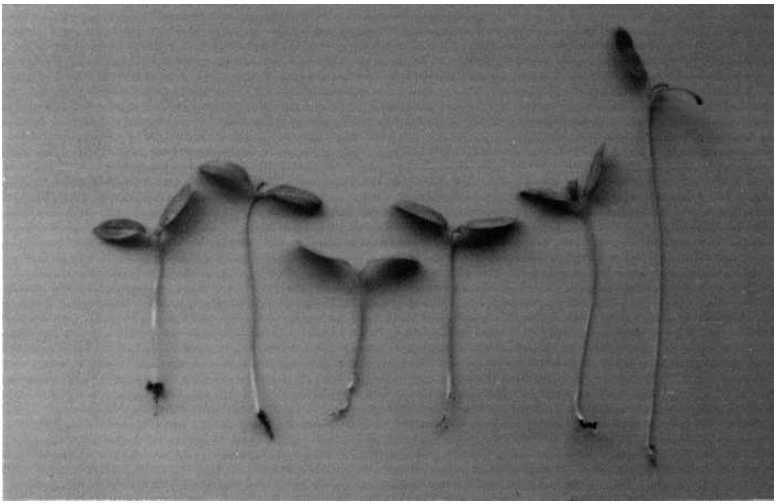

Figure 3.2 Phenotype of 7-day old light-grown light grown mutants in comparison with wild-type seedlings. From left to right: wild type (MM); *fri* mutant; *fri,hp-1* double mutant; wild type (GT); ***tri*** mutant; *trifri* double mutant.

(Koornneef et al., 1985; Parks et al., 1987). Etiolated seedlings show reduced or no inhibition of elongation by red light during de-etiolation, but are significantly inhibited by moderate levels of broad band blue or UV-A radiation (Adamse et al., 1988; Lercari et al., 1991; Peters et al., 1992).

fri {fax red *i*nsensitive): The isolation of this mutant has been described by van Tunenin et al., (1995) which is completely blind to far-red light and shows longer hypocotyl under this condition. It lacks bulk pool of phytochrome (phy A) in etiolated seedlings and immunologically detectable PHYA (Van Tuinen et al., 1995a). *The fri* mutation is thought to be due in the *PHYA* gene itself (Pratt et al., 1997) and evidence has been presented indicating *fri* (Lazarova et al., 1998) is null mutation for PHYA.

hp-1 (*h*igh*p*igment-*1*): This mutation confers an exaggerated phytochrome responses, particularly at the seedling stage: shorter hypocotyl, more anthocyanin and darker green leaves and is thought to identify a gene involved in phytochrome signal transduction (Peters et al., 1992). The mutation shows immature fruit colour due to high chlorophyll levels, higher lycopene and carotene content resulting in deep-red fruits (Kerckhoffs et al., 1997b).

tri (*t*emporarily red light insensitive): This mutant was isolated by van Tuinen et al., (1995b), for its longer hypocotyl under white light and blind to red light. The mutation has been shown to be due to the *PHY*B1 gene itself (Kerckhoffs et al., 1996; van Tuinen et al., 1997; Pratt et al., 1997).

3.3 DOUBLE MUTANT LINES

tri,fri: This double mutant is essentially similar to *tri* mutant in WL and essentially blind in the R and FR light (Kendrick et al., 1997).

au,hp-1: The construction of the double mutant was described by Adamse et al., (1989). This double mutant is deficient in bulk light-labile phytochrome pool, has a phenotype closer to *au* than *hp-l* (Kendrick et al., 1997).

fri,hp-1: This double mutant phenotypically resembles close to *hp-l* (Kendrick et al., 1997).

3.4 SEED GERMINATION AND SEEDLING GROWTH CONDITIONS

For chloroplast development studies seeds of both *au* and wild type were surface sterilized with 1.5 % commercially available sodium hypochlorite, washed thoroughly with distilled water and dried on filter paper. Seeds were then spread on 0.7% agar in 55xl05mm plastic germination boxes. After 4 d of growth in darkness at 25° C the seedlings were grown in continuous white light or red light; cotyledons were harvested at different time intervals and used for further experiments.

For phototropic and gravitropic studies with dark-grown seedlings, seeds of wild type and mutant tomato: *au, hp-1*, and *au,hp-1* double mutant, *fri, tri,* and *fri,tri*, *fri,hp-1* double mutants (*Lycopersicon esculentum* L. cv AC or MM or GT or mixed background) were surface sterilized with 1.5 % sodium hypochlorite for 15 min and rinsed thoroughly with distilled water for 20 min. The seeds were spread out in germination boxes on filter paper moistened with distilled water covered with lid and placed in dark. The germinated seed were picked and transferred individually to 1.5 cm (w) x 1.0 cm (I) screw caps filled with soilrite mixture. All the caps were then placed on moist soilrite mixture in plastic trays and covered with another tray from above. The seeds were

incubated in dark for 24-48 hrs. Seedlings grown having uniform morphology were selected and used for experiments on phototropism and geotropism, less than 50 % of the entire population could be used for the experiments. At the time of phototropic/geotropic stimulation the hypocotyl length was 1.5 - 2.5 cm long and 3-4 days old. In experiments in which norflurazon [4-chloro-5-(methylamino)-2-(α,α,α-trifluro-m-tolyl)-3(2H)-pyridazinone] was used, seedlings were grown on Whatman 3MM paper soaked with a 0.1 mM norflurazon solution.

For phototropic experiments with de-etiolated seedlings, tomato seeds were sown in petri plates filled with wet soilrite. They were germinated and grown for 3 days in continuous white light and de-etiolated at 25° C. The seedlings following various de-etiolating light treatments were used after 2 h dark period for phototropic response.

For seed germination test seeds were sown on a layer of Whatmann filter paper moistened with 2 ml of distilled water. 100 seed were sown in each box and were used for each individual experiment. The boxes with seeds were incubated at 25 °C in the dark or light.

For experiments with roots, seeds of wild type and mutants were soaked in water and then germinated in dark on moist filter paper and after 3 days, by which time the radical had emerged to a length of 5 mm, individual seedlings were transferred to glass vials containing 0.7 % agar and allowed to grown in dark for 1-2 days and then used for phototropism or gravitropism experiments.

For all experiments comparisons between wild type and mutant were always made with seed lots from the same harvest date and were grown side-by-side under the same conditions. All necessary manipulations were carried out in total darkness, except for the photomorphogenic light treatments, and during preparation for gravitropic and phototropic induction. For seedlings grown entirely in the dark, the same conditions of temperature, humidity, and watering were maintained though out the experiment. The temperature in light and dark was maintained at 25°C using a temperature controller (Saveera Biotech, India).

3.5 CHLOROPLAST ISOLATION AND PSII MEASUREMENTS

Freshly harvested cotyledons (200-400 mg) were homogenized for 30 sec in buffer with 1-2 ml of 67 mM phophate buffer (KH_2PO_4/Na_2HPO_4; pH 7.2) containing 0.5 M sucrose, 1 mM $MgCl_2$ and 0.2% BSA. The homogenate was filtered through four layers of cheese cloth into 1.5 ml Eppendreoff tube and first centrifuged at 200 x g for 2 minutes to remove debris and then centrifuged at 3000 x g for five minutes. The pellet was resuspended in the same buffer and the crude preparations were stored at 4 °C until use. Aliquots of sample were removed for chlorophyll estimation and was estimated according to Arnon (1949).

H_20 to DCPIP photoreduction was assayed spectrophotometrically. The light was filtered through 10 cm path of water in round bottom flask to cut off infra-red radiation. A 1 ml quartz cuvette contained the chloroplast preparation (10ug chl/ml), 10 mM phosphate buffer (pH 6.8), 100 mM KCl, 0.1 mM $MgSO_4$ and 20 uM DCPIP. PSII activities were calculated using extinction coefficient of 600 nm as reported by Armstrong (1964).

3.6 PHOTOTROPIC STIMULATION AND RED/FAR-RED PRETREATMENTS

Unilateral blue light was obtained through a cardboard having a slit of 5cm (l)x 1cm (w) by placing in front of the light source. The direction of light was kept perpendicular to the plane of the seedling. Phototropic stimulation with a pulse of unilateral blue-light was given for a duration 10 s or more depending on the study made. The orientation used in pulse induced experiments was such that phototropic stimulus fell perpendicular to the hypocotyl away from the hook. The entire hypocotyl length or apical portion of seedling was unilaterally irradiated for phototropic induction. Dose response or fluence experiments were performed by irradiating the seedlings with the exposure time desired and incubating in darkness until maximum curvature was reached. The fluence rates and exposure times used are given in the description of each experiment. Since the maximum curvature is induced in this direction and not influenced by any shading due to cotyledons. In experiments where light was given continuously, measurements of curvature was made at the intervals indicated and no influence of hypocotyl hook towards blue light source could be detected. The phototropic curvatures were allowed to develop for various time intervals following the start of phototropic induction, and were then measured. In some experiments, vertical irradiation (irradiation from above) with red light or far-red light was carried out prior to the unilateral blue light irradiation.

3.7 GRAVITY STIMULATION

Gravitropism was induced and measured under growth-room conditions in 3-4 day old plants. The hypocotyls were oriented so that the stimulus and response were perpendicular to the plane of apical hook. To the vertically grown roots stimulus was applied by re-orientating the vials through 90° in the horizontal

plane. Following stimulation the curvature of each hypocotyl or root was measured.

3.8 HEAT STRESS TREATMENT

Thermal stress was applied to dark-grown seedlings. The seedlings were imposed to heat stress 42°C or 52°C for different times of exposure (2 min or 5 min) in a hot oven with temperature regulator. Controls were kept at 25°C.

3.9 PHYSIOLOGICAL ASSAYS

For germination assay visible radicle protrusion was used as a criterion for germination. To determine germination frequencies, germinated and non-germinated seed were counted 3 d after the start of germination test.

For assaying root phototropism and gravitropism germinated seed were placed horizontally on 0.7% agar plates and were placed vertically in dark for 24 hours. The plates were then tested for root phototropism or re-oriented so that the roots were perpendicular to gravity.

For assaying interaction of phototropic and gravitropic responses seedlings were simultaneously stimulated for phototropism and gravitropism by orienting the seedling to 90° to the unilateral light coming opposite to the direction of gravity.

For assaying hypocotyl growth curvature induced by auxin, 3 day-old white light grown seedlings were used. The IAA solution (100 μM) was applied to one side of the cotyledon and grown under white light for 24 h before being measured for growth orientation.

For assaying triple response 2-day old dark grown seedlings were grown in the presence of ethylene for 7-days in dark. Inhibition of hypocotyl elongation, gravitropism and radial expansion of hypocotyl growth was taken as indication of action of ethylene.

3.10 MEASUREMENT OF CURVATURE AND HYPOCOTYL LENGTH

For curvature measurements at different time intervals after the beginning of phototropic or gravitropic stimulus the seedlings were removed from vermiculite in cases where a single measurement was to be made and gently placed on a transparent sheet and then photocopied on a xerox machine or *in situ* as in time course experiments. The angle between line to the long axis of the hypocotyl and line to the bending portion was measured using a protractor. This was done by sliding the protractor over the image of hypocotyl, the arc is found to which the curve of hypocotyl fits which was taken as angle of curvature.

Hypocotyl elongation rates were calculated from hypocotyl length measurements taken at the beginning of heat stress treatment and the end of incubation period or after emergence of dark-grown seedlings and incubation for 5 days in dark. The hypocotyl length was measured with a ruler and the distance between the base of hypocotyl and hook or apex to the nearest 0.5 mm.

3.11 MUTAGENESIS AND M_2 SEED COLLECTION

Chemical mutagenesis was performed essentially according to Koornneef et al., (1990). Approximately 5000 seeds of *Lycopersicon esculentum* cv. Alisa criag were soaked in distilled water containing 60 mM ethyl methane sulphonate for

24 hours and incubated in dark at room temperature. During incubation the seeds were mixed occasionally. Afterwards, the seeds were thoroughly washed 10-15 times with distilled water for 2-3 hours and the M_1 generation seed obtained after chemical mutagen treatment were germinated in pots and, grown in the field. The M_1 plants were allowed to self and the seed was harvested in bulk from fruits collected (M_2 generation). This M_2 seed was used for the subsequent screening steps.

3.12 SCREEN FOR MUTANTS

Dark grown seedlings raised from M_2 seed were analyzed for phototropic response. Approximately 200-300 seedlings were analyzed simultaneously on a single petri dish. The plates were treated to unilateral blue light or white light for 12-16 hours. Only mutants that did not bend towards light were selected. These putative mutants were allowed to green in white light for a week before being transferred to pots and allowed to mature and set seed under natural growth conditions. M_3 seed was collected from each of the M_2 plant and re-screened for phototropic response and finally selected in M_4 and M_5 generations.

3.13 GENETIC CROSSES AND CHARACTERIZATION

Crosses were made by emasculation of the flowers before opening of the corolla, immediately followed by pollination with different pollen. After completion of pollination the flower was labeled. F_1 plants from crossings were grown to maturity or used for getting BC_1 generation. The progenies of these plants were analyzed. The F_1 seedlings and segregation ratios in F_2 seedlings were determined by counting the number of seedlings with or without phototropism towards unilateral blue light.

3.14 LIGHT SOURCES AND MEASUREMENTS

For chloroplast development studies a strong source of light, for monitoring DCPIP photoreduction by PSII complexes, was obtained by 300W slide projector. Red light was obtained from two 40 watt coolwhite fluorescent tube light filtered through two layers of red plexi glass sheets. White light treatments were by four 30 watt coolwhite flourescent tube lights.

For phototropism experiments continuous unilateral source of blue light was obtained by two 20 watt fluorescent tube light filtered through CBS 450 blue filter (Carolina Biological Supplies, USA). Unilateral red light was obtained by two 20 watt fluorescent tube lights and a CBS red filter. Continuous sources of white light and UV-light were obtained by one 20 watt fluorescent tube and two 15 watts (G15 T8 Philips, Holland) UV lamps respectively. A glass plate of 4 mm thick was used in UV-light phototropism experiments to filter the unwanted rays.

Unilateral monochromatic blue light for pulse induced phototropic experiments was obtained by passing light from a projector (Philips 150 W lamp) through a CBS blue filter. Red light or Far-red light for phototropic pre-treatments was obtained by passing light from 150 W Philips Lamp through a 2-cm layer of water and a CBS red or far-red filter. Photon fluence rates of blue, red, far-red and white light were taken with a model light meter fitted with a quantum sensor (Skye instruments, UK).

For handling dark-grown seedlings at the time of photomorphogenic light treatments, and during preparation for phototropic/gravitropic induction, **dim** green safe light was obtained through eight layers of green cellophane paper

from a 40 watt coolwhite fluorescent lamp. The fluence rate of this light at the plant was no greater than 0.001 μmole m^{-2} s^{-1} at any time, and lasted no longer than 2-5 min. The green light radiation applied unilaterally for periods of up to 5 min, induced no detectable phototropic curvature.

3.15 STATISTICS

Wherever necessary experiments were repeated at least thrice or more with qualitatively and quantitatively similar results and are presented as the final mean value ± S.E or S.D.

CHAPTER 4

RESULTS

During de-etiolation it appears that both blue light absorbing photoreceptor and phytochromes are involved in the regulation of phototropism (Liscum and Stowe-Evans, 2000). At present we have relatively little physiological information on phytochrome involvement in phototropism in part because the blue/UV-A photoreceptor assumes much greater importance in this response (Liscum and Briggs, 1995; Huala et al., 1997). There appears to be interactions between these photoreceptor systems as well, but the nature of it has not yet been fully elucidated (Liu and Iino, 1996; Janoudi et al., 1997). Aside from this, one or more signal response chains are likely required between a photoreceptor and its final effect on the response, however, the pathways of which are also partially known (Parks et al., 1996; Janoudi et al., 1997). Furthermore, some sort of amplification system seems necessary to account for the response and rather little is known about the mechanisms involved in the phototropic response.

The most extensive photobiological studies on phototropism have been carried out with eitolated seedlings (Iino, 1990). The phytochrome responses, which offer a well-characterized photoreceptor system and the blue light photoreceptor are often more prominent in etiolated material. Even such an apparently simple system of phototropic bending exhibits a great diversity of responses. These responses can differ with respect to light requirement, time course, and induction kinetics. However, most of our knowledge of phototropism in higher plants has come from experiments on coleoptiles of monocot seedlings, particularly those of *Avena sativa* and *Zea mays* (Iino, 1990) and in recent years on hypocotyls of dicot plant *Arabidopsis thaliana* (Liscum and Briggs, 1995). These coleoptiles or hypocotyls have the advantages for studies of phototropism for anatomical simplicity, uniformity

and rapidity of growth, high phototropic sensitivity, and fast response to phototropic stimuli. Nevertheless, the detailed studies of phototropism in other higher plants would also be useful to extend to other genera before generalizations can be made about light perception and transduction, and the relation of these to the eventual response.

Tomato is an ideal system for examining light regulation of phototropism since it has been extensively used in the study of photomorphogenesis (Kendrick et al., 1997). This particular species is known to respond to both light absorption by phytochrome and blue/UV-A photoreceptor. The phytochrome gene family has been characterized and to date five phytochrome genes have been identified: PHYA, PHYB1, PHYB2, PHYE and PHYF (Hauser et al., 1995). However, there is little information available on the characteristics of blue/UV-A light photoreceptor (s) in tomato seedlings. Thus, putative mutants for blue light photoreceptor can be isolated in tomato as it is a diploid and an ideal system for classical genetic studies and can be cultured easily under wide range of environmental conditions.

In this study, for the above mentioned reasons tomato was chosen as an experimental material to characterize phototropism. Phototropism has not yet been studied in detail in tomato, therefore, in the first part of the study basic physiological data on the phototropism of tomato hypocotyls was obtained. Additionally we have used another approach to understand how photoreceptors interact in phototropism by studying the response of mutants that are altered in their response to one or more of these factors. This approach further helps to define components involved in a complex response and for studying the signal transduction pathways that link an activated photoreceptor with a response. To

this end, two sort of photo-responsive mutants were examined: phytochrome deficient mutants with altered photomorphogenic responses *viz.,* the chromophore deficient *au*, the phyA deficient *fri,* the phyB1 deficient *tri* and the *tri fri* mutants. The second type of response mutant examined, *hp-1*, shows opposite phenotype exhibiting exaggerated photomorphogenic responses to light and its interaction in *au,hp-1* and *fri,hp-1* mutants was assessed to clarify the role of *hp-1* in phototropic response. Furthermore, another approach to study phototropic sensory transduction involves isolation of mutants with an altered sensitivity to light. A screening technique for mutants that would exhibit no phototropism in constant light was developed. A genetic and physiological characterization of two such mutants, which have been isolated, is presented here.

4.1 PHOTOTROPISM IN TOMATO

As a preliminary to studies of light induced changes in phototropic response, we tested the effect of unilateral blue light and red light and determined the time course of response induced in tomato seedlings. The dark-grown seedlings were subjected to continuous unilateral blue light (450nm) for 120 min. The course of phototropic curvature induced during the 120-min measurement period is presented in Fig. 4.1. The phototropic curvature is manifested after a lag time of about 30-45 min from the onset of illumination and the initiation of the response. Thereafter, the curvature increased to about 64° from the vertical, theoretically roughly parallel to the direction of the light stimulus for the next 90 min. However, the curvatures began to decrease during the final assay period, as is typical when there was self-adjustment of response to light. On the contrary, seedlings irradiated with unilateral red light displayed no curvature (Fig. 4.1). Thus, blue light elicited maximal bending

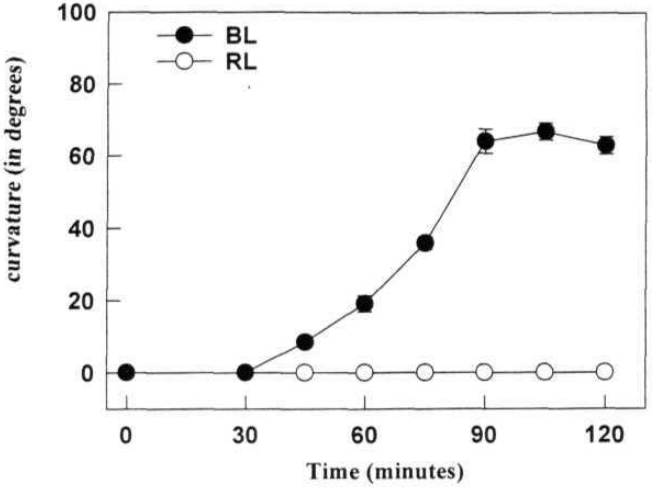

Figure 4.1. Time course of phototropic curvature (degrees) of tomato hypocotyls to unilateral blue (0.1 μmole m^{-2} s^{-1}) or red light (0.3 μmole m^{-2} s'"). Seedling curvature was determined at the time points indicated. The data point represent mean value ±S.E of three independent experiments using 10 dark-grown seedlings per set.

angles, while red light was ineffective in inducing phototropic response in tomato.

To determine the dose-response relationship the phototropic response of dark-grown hypocotyls was determined. Dark grown hypocotyls were irradiated with single pulse unilateral blue light of 0.01 $\mu mol\ m^{-2}$ s'" for varying duration from 1-120 sec. The direction of growth was determined 2 hrs after the dark incubation. It can be seen from Fig. 4.2 that the phototropic curvature of tomato hypocotyls exhibits two peaks of curvatures. According to terminologies used (Iino, 1990) the first peak of curvatures induced by a pulse of blue light represent first positive response and second peak of curvatures represent the second positive response. The first peak shows the bell-shaped appearance with a lower threshold at about $0.1 \mu mol\ m^{-2}$, a maximum of more than 10° at 1 $\mu mol\ m^{-2}$ and zero curvatures at fluences higher than 10 $\mu mol\ m^{"2}$. This peak is followed by zone of indifferent curvatures where no response could be manifested. The second positive phototropic curvatures are initiated after longer exposure times. It is initiated at an exposition time of about 10 min and increased with increase in fluence.

4.2 INFLUENCE OF HOOK POSITION ON PHOTOTROPISM

Since position of hook with respect to direction of light has a profound effect upon hypocotyl curvature, the orientation of seedlings was determined to know the magnitude of curvature induced. The dark-grown hypocotyls were irradiated unilaterally with a 10-sec pulse or 2-hrs continuous blue light by varying the orientation of seedling hook to the direction of light. Table 4.1 shows that when hypocotyls of seedlings facing hook towards light source were irradiated unilaterally with a pulse of blue light there was no curvature. In

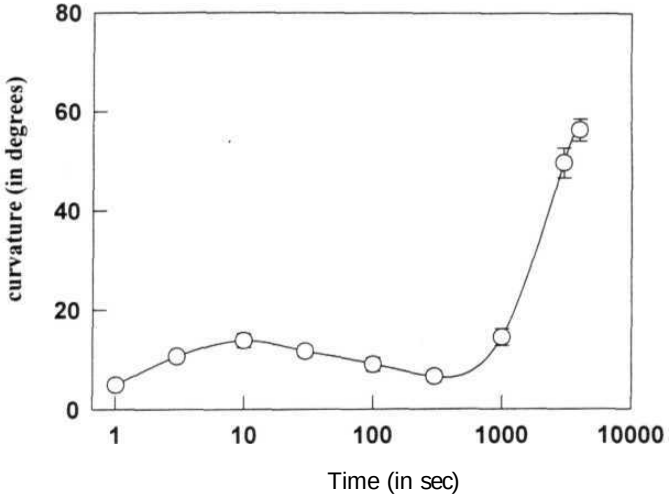

Figure 4.2. First and second positive phototropism in hypocotyls of dark-grown tomato seedlings. The seedlings were exposed to a single blue light (0.1 μmole m^{-2} sec^{-1} X = 450 nm) pulse of varying duration indicated on ordinate and were returned to darkness for 90 min, when curvature was determined. The data points represent mean value obtained for 30 seedlings and error bars indicate standard error of mean.

Table 4.1. Influence of hook position and cotyledons on the phototropic response of tomato seedlings. The average maximum curvature was determined for 30 seedlings per treatment after phototropic stimulation for 10 sec or 2-hrs unilateral blue light. Variation is expressed as mean ± S.E

Response type	Influence of hook		Influence of cotyledons
	Hook away from direction of light	Hook in the direction of light	
First positive	14.3° (±1.1)	1.0° (±0.4)	12.3° (±1.3)
Second positive	88.3° (±3.0)	58.0° (±2.9)	59.4° (±2.4)

contrast on exposing seedling with hook away from light source a positive curvature of about 14.3° was induced. On the other hand, seedlings exposed to continuous blue light the degree of curvatures were smaller when the hook faced towards the light source. In contrast, when hook faced away from the direction of light source the observed angles of curvatures, however, were larger. Thus maximal angles of curvature were induced in seedlings hook facing away from the direction of light source and minimum angles were found in seedlings hook facing towards the direction of light source.

4.3 EFFECT OF COTYLEDON REMOVAL ON PHOTOTROPISM

To investigate whether the perception of the phototropic stimulus is located in the hypocotyl itself, or cotyledons, the cotyledons of tomato seedlings were surgically removed immediately before unilateral illumination. The decapitated seedlings and control seedlings were stimulated to continuous unilateral blue light for 2 hrs and response determined. The results show those hypocotyls of cotyledons removed are found to be competent to execute both first and second positive phototropic curvatures (Table 4.1). Since there is no statistically significant difference in curvature with reference to the intact control plants, indicating that hypocotyl itself can perceive light stimulus.

4.4 EFFECT OF HEAT STRESS ON PHOTOTROPISM

The effect of temperature stress was studied on phototropism and gravitropism of tomato seedlings. Fig. 4.3 shows that the dark-grown seedlings at room temperature (controls) show normal phototropism and gravitropism and when subjected to heat shock treatment for 2 min at 42°C or 5 min at 52°C temperature there was no response. However, when the seedlings after heat shock treatment were returned to room temperature for 24 hrs in dark and

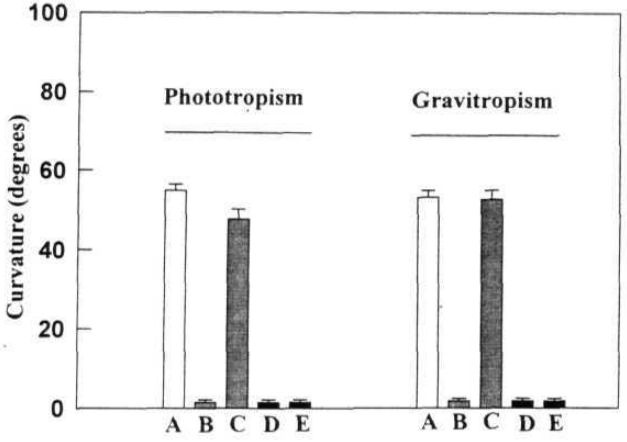

Figure 4.3. Effect of heat stress on phototropic and gravitropic growth of 4 day-old, dark grown tomato seedlings. Plants were exposed to either 42° C for 2 min (B) or 52° C for 5 min heat stress (D) which were immediately stimulated to phototropism or gravitropism at room temperature and compared to untreated seedlings (A). A set of seedlings were returned to darkness for 24 hrs at room temperature and then subjected to phototropism or gravitropism (C and E). The phototropism was induced by exposing seedlings to continuous blue light and for gravitropism seedlings were oriented perpendicular to gravity direction. Measurement of curvature was performed 2 hrs after the phototropic or gravitropic stimulus treatment. Error bars represent S.E of mean.

stimulated to phototropism and gravitropism, the responses recovered in 2 min treated samples at 42° C but not in 5 min treated samples at 52° C.

Since the tropic responses are known to depend on the growth changes, the effect of heat stress was studied on hypocotyl growth. The heat stress was given to hypocotyls and the increase in hypocotyl length was measured. Fig. 4.4 shows that the observed loss of phototropism and gravitropism in heat shock treated seedlings was related to heat-mediated inhibition of the hypocotyl growth. While the hypocotyl growth recovered in seedlings treated to 2 min heat shock during subsequent incubation at room temperature in dark for 24 h. By contrast, seedlings exposed to 5 min heat shock did not regain growth even after 24 h incubation at normal room temperature. Thus at higher temperatures, perception of light or gravity is disrupted as a consequence of growth inhibition.

4.5 INFLUENCE OF GRAVITY ON PHOTOTROPIC RESPONSE

In the first set of experiment the effect of gravity on phototropic growth was investigated by treating seedling to gravity prior to phototropic stimulus. The seedlings after germination were grown for 3 days in dark and stimulated to gravity for 20 min by orienting seedlings perpendicular to gravity and treating to continuous unilateral blue light for 2 hrs. In another experiment the effect of gravity was investigated by orienting seedlings perpendicular to gravity and placing the unilateral blue light source in the direction of gravity to treat simultaneously to phototropic and gravitropic stimuli. No significant decrease in phototropic curvature could be detected in both the cases (Fig. 4.5). This result indicates that phototropic response supercedes gravitropic response.

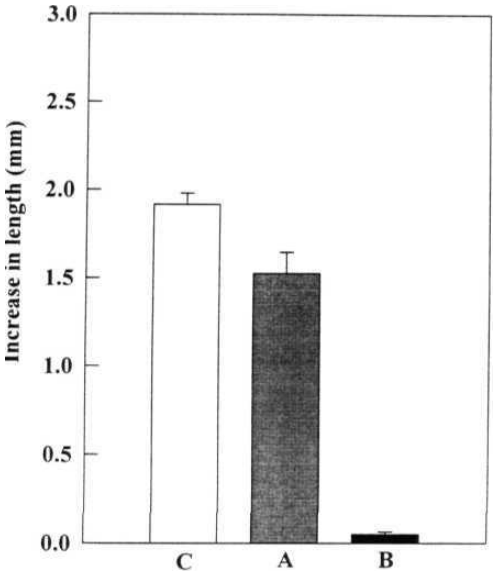

Figure 4.4. Effect of heat stress on hypocotyl growth in 4-day old dark-grown seedlings. The seedlings were exposed to either 42° C for 2 min (A) or 52° C for 5 min (B) thereafter seedlings were incubated in dark at 25° C for 24 hrs followed by measurement of increase in length of hypocotyl. Controls were incubated at room temperature (C). Each histogram bar represent mean of at least 30 seedlings and error bars represent S.E of mean.

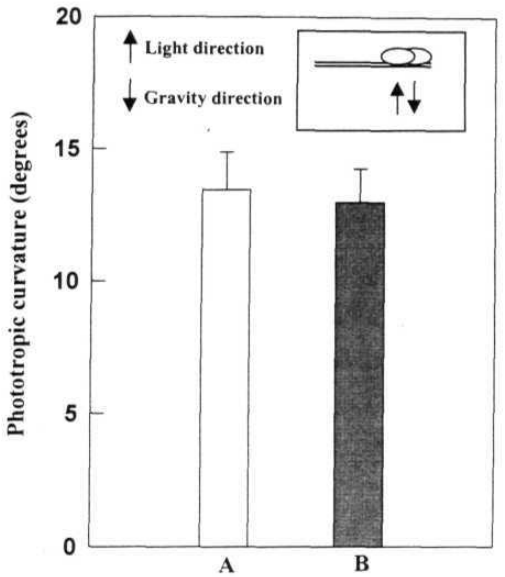

Figure 4.5. Interaction between phototropism and gravitropism on curvature of tomato hypocotyls. One set of seedlings were orientated perpendicular to direction of gravity for 20 min in dark and then treated to phototropic stimulation (A). Another set of seedlings were simultaneously treated to gravity and phototropic stimuli (B) by exposing to unilateral blue light source coming in the opposite direction of gravity (inset). Curvature was measured after 1 hr of onset of light.

4.6 EFFECT OF CAROTENOID BLEACHING ON PHOTOTROPISM

Over the years carotenoid has been proposed to be the chromophore responsible for phototropism. One of the carotenoid, zeaxanthin, was shown to be involved in the perception of phototropic signal in maize (Quinones et al., 1996 ; Quinones and Zegier 1994). To assess the relative role of carotenoids in phototropic response the seedlings were treated to Norflurazon, which interferes with the desaturation of *cis*-phytoene thus blocking the accumulation of carotenoids. The seedlings germinated in the presence of Norflurozan showed a substantial reduction in the accumulation of carotenoid levels (Fig 4.6). However, when the control and Norflurazon bleached seedlings were treated to unilateral continuous blue light for 2 hrs, Norflurazon treated seedlings had a normal phototropic response, although these seedlings showed less curvature than control seedlings, (Fig 4.6).

4.7 EFFECT OF RED OR FAR-RED LIGHT PRE-TREAMENTS ON ENHANCEMENT OF PHOTOTROPISM

Although phototropism is a blue-light mediated response, pre-irradiation with red light enhances the phototropic response. The effect of red light pre-irradiation on blue light induced first positive response was examined by comparing effect of ominilateral pre-irradiation of red light from above for 5 min and incubated in dark for 90min prior to the blue light pulse. Table 4.2 shows magnitude of curvature determined 90 min after the blue light pulse treatment. In non-preirradiated seedlings a curvature of 10.2° was observed. However, in seedlings that are pre-irradiated with red light there was one-fold increase in the response of about 20.8°. The red/far-red reversibility of phototropic enhancement was tested by following 5 min red light preirradiation with 5 min far-red irradiation. As shown in the Table 4.2. the red light

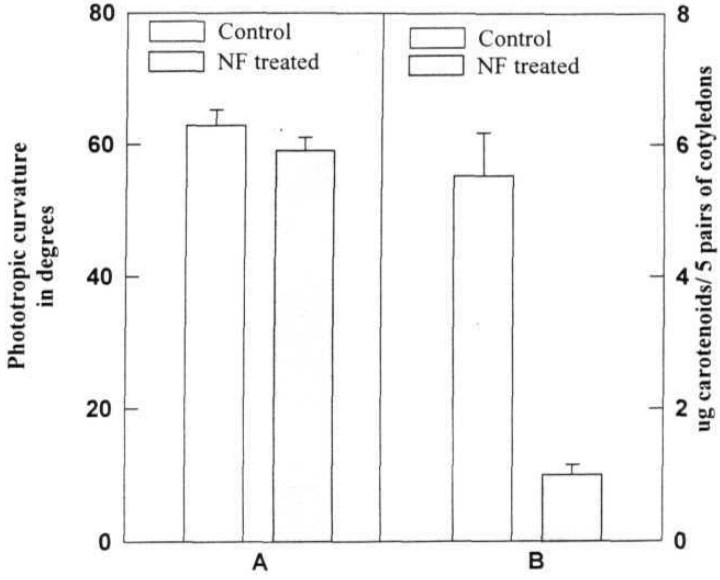

Figure 4.6. Effect of norflurazon treatment on phototropic curvature (A) and accumulation of carotenoids in red-light grown seedlings (B). The seedlings were germinated in the presence of norflurazon or distilled water (control) and treated to phototropic stimulus for 2 hrs (A). Carotenoid content of norflurazon treated seedlings was determined from the absorbance at 473 nm of cotyledons of 5 seedlings and compared to that of control (B). Error bars represent S.E of

Table 4.2. Red light induced enhancement of fist positive phototropism, the extent of far-red reversibility and synergism of red and far-red light in hypocotyls of tomato. Average maximum curvature was determined for 30 seedlings per treatment after phototropic stimulation for 10 sec and incubation in dark for 90 min. R= 25.21 $\mu mol\ m^{-2}\ s^{-1}$ at λ= 660 nm; FR=21.2 $\mu mol\ m\ \ s$ at λ= 735 nm.

Treatment	First positive phototropic curvature in degrees
D	10.2 (±3.6)
R	20.8 (±1.8)
R/FR	20.5 (±1.7)
FR	16.7 **(±1.6)**
FR/R	19.06 (±1.6)

mediated enhancement could not be reversed by a subsequent far-red light treatment. Moreover, when far-red light was given alone rather increased the phototropic curvature to about 16.7°. It is evident from results presented that the response lacks classical red/far-red reversibility as well as far-red light has nearly the same effect.

The effect of red light pre-irradiation was also examined on the time threshold of second positive response of tomato hypocotyls. Dark grown seedlings were pre-irradiated for 5 min with red light and incubated in dark for 90 min. After which, the seedling were exposed to varying duration of time with unilateral blue light at 0.01 $\mu mol\ m^{-2}s^{-1}$. Fig 4.7 shows that non-pre-irradiated seedlings required a time threshold of about 10-min to induce curvatures. In contrast pre-irradiation of seedlings with red light, displayed higher sensitivity to phototropic stimulus by shortening of the time threshold to 5 min and significant increase of magnitude of curvature.

4.8 EFFECT OF BLUE OR WHITE LIGHT PRE-TREATMENTS ON FIRST POSITIVE PHOTOTROPISM

The effect of blue or white light pre-irradiation followed with red or far-red light were examined on first positive phototropic response by treating immediately after pre-irradiations with unilateral blue light pulse for 10 sec at 0.01 $\mu mol\ m^{-2}\ s^{-1}$ and determining curvature after 90min incubation in dark. Fig. 4.8. shows that first positive response could be detected only in dark-grown, red light or far-red light pre-treated seedlings. In contrast, first positive response significantly declined in seedlings pre-treated with either white light or blue light. Interestingly, the blue light pretreatment followed by red or far-

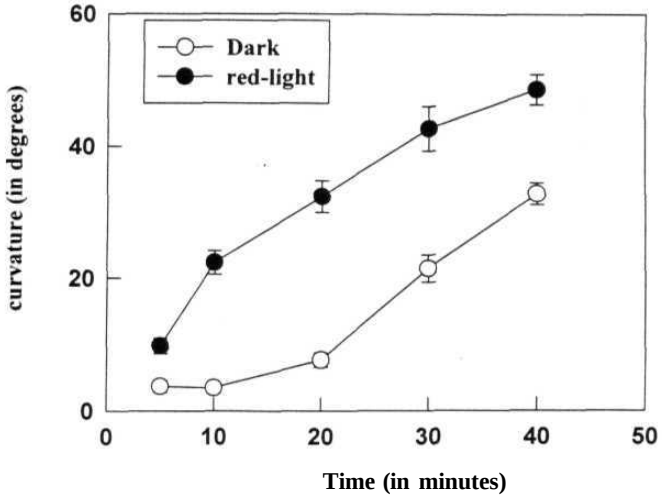

Figure 4.7. Effect of red light on time-threshold of second positive phototropism in tomato hypocotyls. Dark grown seedlings were pre-treated to 5 min red light (fluence rate = 25.21 μ mole m^{-2} s') and incubated in dark for 90 min. Thereafter the seedlings were treated to blue light pulse for the duration of time indicated on abscissa. Curvature was measured 90 min after the start of stimulus treatment. Each data point represents mean of 30 seedlings and error bars indicate S.E of mean.

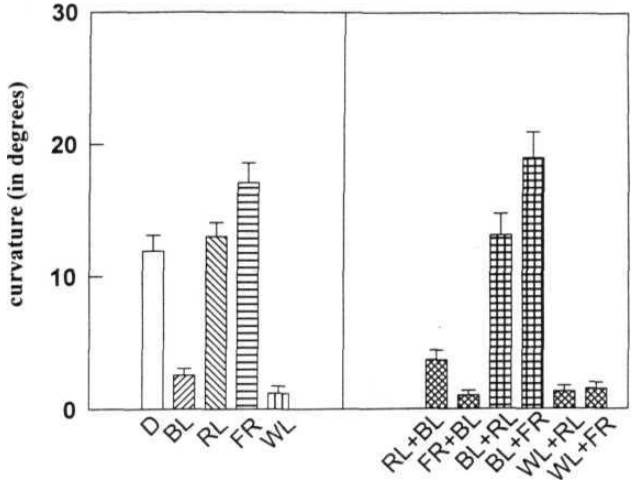

Figure 4.8. First positive response of tomato hypocotyls to various light treatments. 5 min BL , RL, FR or WL, 5min BL + 5min RL or FR and 5min WL + 5 min RL or FR were given as pretreatment followed by unilateral blue light for 10 sec and curvature was measured after incubation for 90 min in dark. The bars represent mean value ± S.E. Ten seedlings were used for each experiment, which was repeated three times for each point.

red light restored the first positive response, whereas white light pretreatment followed by red or far-red light could not restore the response.

4.9 KINETICS OF FIRST AND SECOND POSITIVE PHOTOTROPISM DURING DE-ETIOLATION

Only limited information is available for the sensitivity of seedlings to show first and second positive responses during de-etiolation. After germination in dark the seedlings were grown under ominilateral white or red or blue light, and the first positive response was monitored during the course of de-etiolation for 8h when the seedlings just opened the hooks. Fig. 4.9 shows that first positive response was lost in white or blue light treated seedlings. The response is retained and also enhanced in red light-grown seedlings (Fig. 4.9). Similarly, the kinetics of second positive response was also monitored during de-etiolation of seedlings. Seedlings grown under continuous white light was examined for occurrence of second positive response at regular intervals of 24 hours. Fig. 4.10 shows that the seedlings responded to second positive stimulus during early phase of seedling emergence 1, 2 or 3-day-old seedlings, but the sensitivity is gradually lost as seedlings aged. This indicates that although second positive phototropism is retained during early stages of de-etiolation the response is lost in completely de-etiolated seedlings.

4. 10 SECOND POSITIVE PHOTOTROPISM IN *au* AND//-/ MUTANTS

If phytochrome plays a role in the phototropism of tomato seedlings, then mutants with altered in phytochrome apoprotein or its chromophore should show modified response. The availability of phytochrome photoreceptor mutants enables the characterization of its role in the co-action with blue/UV-A photoreceptors to regulate phototropism. Alternatively, physiological

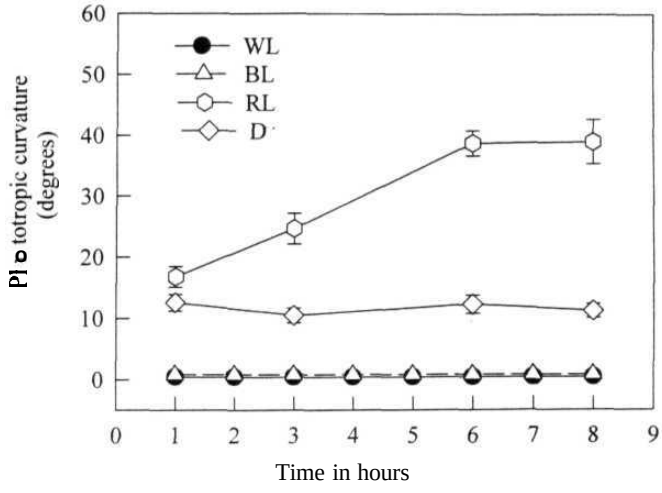

Figure 4.9. The effect of red light (RL), blue light (BL) and white light (WL) on kinetics and magnitude of first positive phototropic response in tomato seedlings. Fluence rates: RL = 0.3 μmole m^{-2} s^{-1}; BL = 0.01 μmol m^{-2} s^{-1} ;WL=100 μmol m^{-2} s'. The seedlings were removed at indicated time intervals and treated for 10 sec unilateral blue light pulse followed by measurement of curvature after maximal development in dark for 90 min. All data points represent mean ± S.E. Each data point is mean of 30 seedlings.

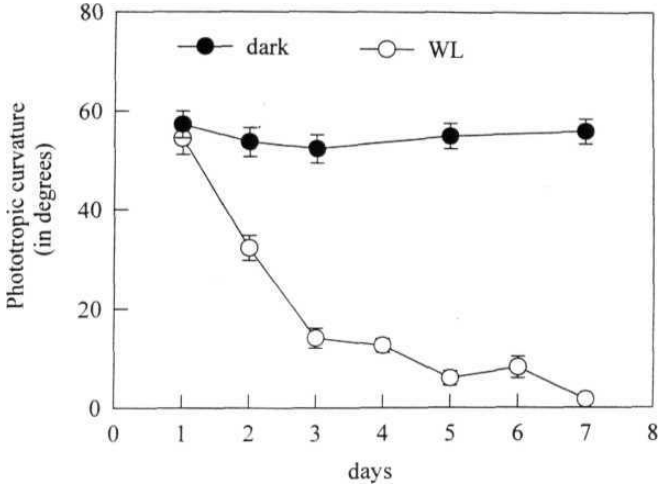

Figure 4.10. The second positive phototropic response of hypocotyls with days after germination in dark-grown or white light (100 μmol m^{-2} s^{-1}) grown seedlings. The white light grown plants were incubated in dark 2 hrs before initiation of phototropic reaction. The phototropism was induced with a unilateral white light irradiation. Experiments were repeated on three occasions using ten seedlings for each assay. The points and vertical lines represent the mean value S.E respectively.

characterization of the known phytochrome mutants may provide insight into the role of phytochrome in phototropism.

Since *au* mutant (possessing < 5% levels of phyA) that is deficient in chromophore biosynthesis (Terry and Kendrick, 1996) has reduced phytochrome level of all phy species, the role of phytochromes in phototropism was analysed using this mutant. The dark grown wild type and mutant seedlings were tested for phototropism by inducing the response with continuous blue light for 6 hr time interval. Fig. 4.11 shows time course of the phototropic curvature in wild type and *au* mutant seedlings. In wild type seedlings exposed to uni-directional continuous blue light, response was induced after 45 min and maximal phototropic curvature was reached by 120 min. In contrast, seedlings of *au* mutant showed insignificant curvature up to 3 hrs and latter sluggishly developed some curvature. Even after 6 hrs the curvature induced in *au* mutant was lesser than wild-type (Fig 4.11).

To further analyze the role of phytochrome in the *au* mutant phototropic response of seedlings were also examined to their sensitivity to UV-A and white light induced phototropism. The second positive curvatures were compared in wild type and *au* mutant after irradiating either with continuous UV-A or white light. While the wild type seedlings showed significant second positive response, the same treatments resulted in insignificant response in *au* mutant (Fig 4.12).

Since the dark grown *au* mutant seedlings had an altered phototropic response, we decided to screen for phytochrome species-specific mutants. **At first, the** role of specific phytochrome species on phototropism was examined

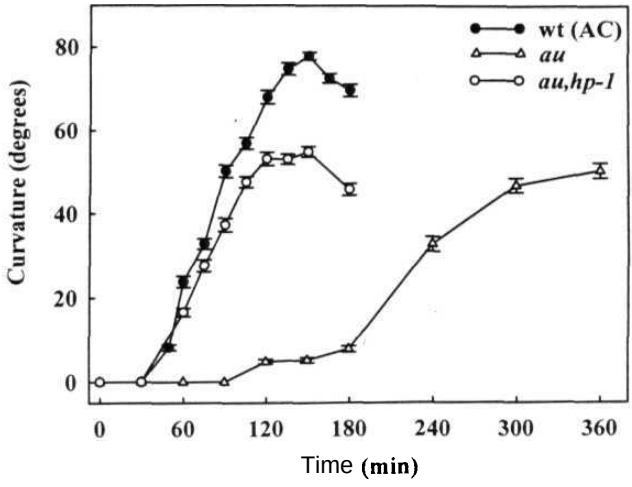

Figure 4.11. Time course of induction of phototropic curvature in wild type and mutant seedling exposed to continuous unilateral blue light at fluence rate of < 0.01 μmol m^{-2} s'. At the time intervals indicated, the seedlings were removed and curvatures were measured. The vertical bars indicate ± S.E of mean and 80 seedlings were measured for each point.

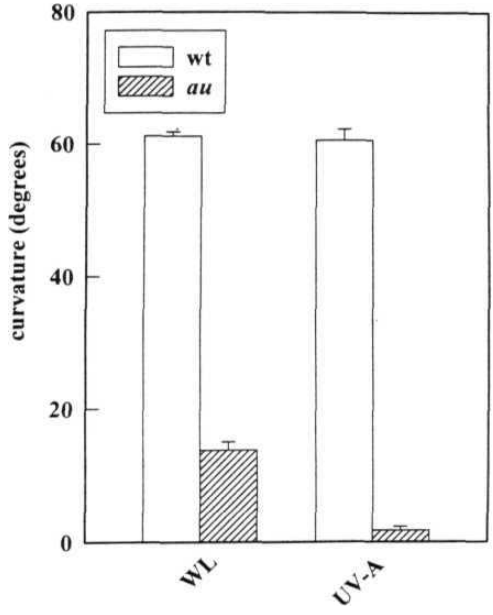

Figure 4.12. Phototropism of 3-day old dark-grown wild type and *au* mutant seedlings to white light (WL) and UV-A light sources. During each exposure 15-20 seedlings were exposed and mean was taken for 12 seedlings. Data represent mean for n=8 such independent determinations. Error bars represent the ± S.E of mean.

by using phy A mutant, *fri.* The *fri* mutant of tomato lacks bulk pool of phytochrome in etiolated seedlings, predominantly phyA (van Tuinen et al., 1995a), as a consequence of mutation in the *PHYA* gene (Pratt et al., 1997; Lazarova et al., 1998). Fig. 4.13 shows comparison of phototropism in wild type and//-/ mutant seedlings to continuous unilateral blue light for 6 hrs. In dark grown wild type (MM) seedlings phototropic curvature was manifested with in 45 to 60 min of stimulation and reached a maximum by 120 min. On the contrary, however, seedlings of *fri* mutant showed no appreciable curvature towards continuous blue light during first 3 hrs and bending is only relatively slight even after 360 min (Fig. 4.13). These observations of phototropic response in *au* and *fri* mutants indicates that both the mutants are severely impaired in second positive phototropism.

4.11 SECOND POSITIVE PHOTOTROPISM IN *au,hp-1* AND *fri,hp-1* DOUBLE MUTANTS

While experiments with *au* and *fri* mutant indicated a role for phytochrome in second positive response, we examined its interaction with *hp-1* mutation in the digenic mutants *au,hp-1* and *fri,hp-1*. Double homozygous mutant lines combing *au* and *fri* with *hp-1* have already been described (Kendrick et al., 1997). As shown in Figs. 4.11 and 4.13, both *fri,hp-1* and *au,hp-1* double mutants showed 45 - 60 min lag period and maximal curvature by 2 h similar to wild type. This indicates that *hp-1* mutation reverses second positive phototropic response to continuous blue light in the double mutants *au,hp-1* and *fri,hp-1*.

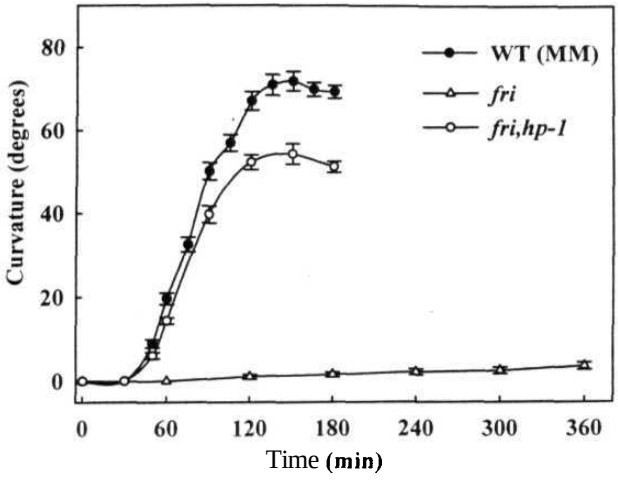

Figure 4.13. Time course of induction of phototropic curvature in wild type and mutant seedling exposed to continuous unilateral blue light at fluence rate of < 0.01 umol m^{-2} s^{-1}. At the time intervals indicated, the seedlings were removed and curvatures were measured. The vertical bars indicate ± S.E of mean and 80 seedlings were measured for each point.

4.12 SECOND POSITIVE PHOTOTROPISM IN *tri* MUTANT AND *trijri* DOUBLE MUTANTS

Possible participation of phytochrome Bl and it interaction with phytochrome A in phototropism the response was investigated by the use of *tri* mutant, and *trijri* double mutant respectively. The *tri* mutant has reduced levels of phytochrome Bl (phyB) and is due to consequence of a mutation of *PHYB1* gene itself (Kerckhoffs et al., 1996; van Tuninen et al., 1997; Pratt et al., 1997). The dark grown seedlings were treated to continuous unilateral blue light for 3 hrs and curvature determined. The seedlings of the phyB1 mutant *tri* showed phototropic response similar to wild type (Fig 4.14). Thus, the absence of phyB1 did not significantly effect the blue light induced curvature. However, the *trijri* double mutant lacking phyA and phyBl behaved similarly *to fri* mutant, showing little or no response. It is evident from this data that the *tri* mutant shown normal second positive response while *trijri* is impaired in the second positive phototropism.

4.13 FIRST POSITIVE PHOTOTROPISM IN *au* AND *fri* MUTANTS

Since the *au* and *fri* mutants are impaired in second positive phototropism, the mutants were tested for first positive phototropic response. The dark grown seedlings of wild type and mutants were treated with a pulse of blue light for 10 sec at fluence 0.01 $\mu mol\ m^{-2}s^{-1}$ and curvature was determined after 2 hrs incubation in dark. Fig. 4.15 and 4.16 show that dark grown seedlings of genotypes AC and MM show a mean curvature of 16.9° and 15.7° respectively. Moreover, the seedlings show a uniform distribution of curvatures with minimum curvatures of 20° to maximum curvatures of 80°. In contrast, *au* and *fri* mutants show a mean first positive curvature of 2.37° and 2.45° respectively (Figs. 4.15 and 4.16) and the frequency distribution shows angles

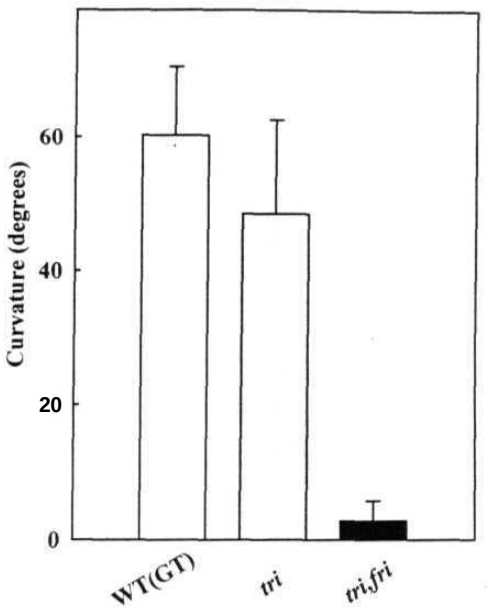

Figure 4.14. Phototropism to continuous blue light in wild type and mutant seedlings. 3-day old dark-grown seedlings were treated to unilateral blue light for 3 hours and curvatures measured. Each histogram represent mean of at least 80 seedlings, the error bars represent S.D of mean.

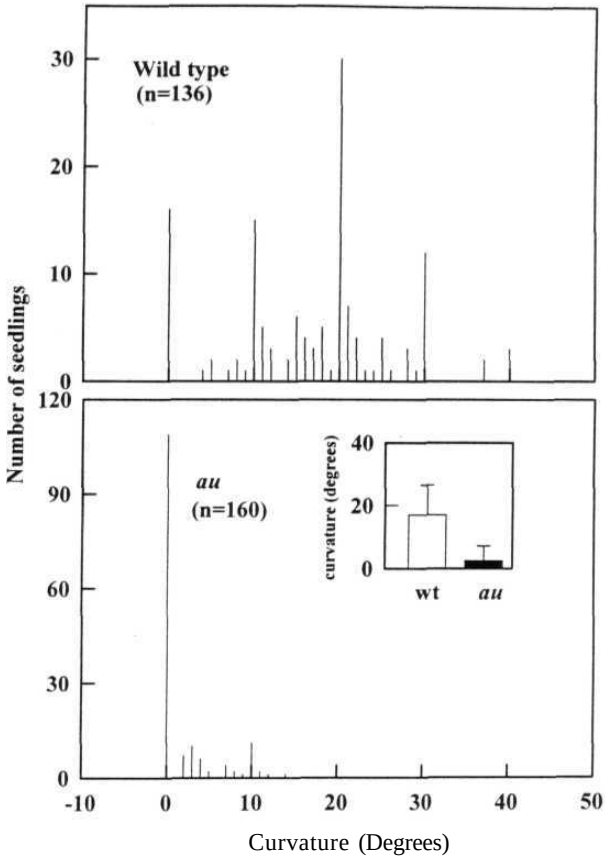

Figure 4.15. Frequency distribution of first positive phototropism in wild type and *au* mutant seedlings to a single pulse of blue light. The 3-day-old dark-grown seedlings were exposed to a single pulse of blue light (450 nm) at fluence 0.1 umol m^{-2} s^{-1} for 10 sec and returned to darkness. Curvatures induced were measured 2 h after the stimulus treatment. The inset shows comparison of mean value (± S.D) of wild type and mutant.

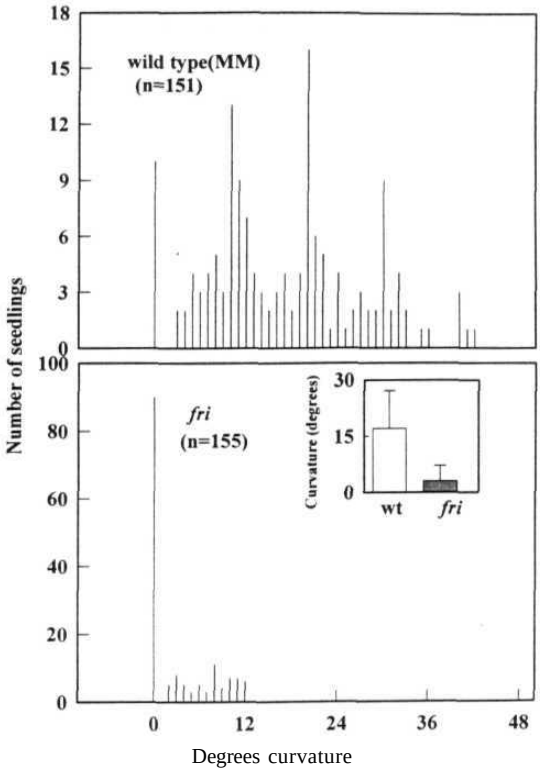

Figure 4.16. Frequency distribution histogram for first positive phototropism showing comparison of wild type and *fri* mutant seedlings. The 3-day-old dark-grown seedlings were exposed to blue light (450 nm) at fluence for 10 sec and returned to dark. Curvatures induced were measured 2 h after the light pulse treatment. The inset shows comparison of the mean curvature value (±S.D) of wild type and mutant.

of curvatures between 0° and 20°. Thus, the results indicate that *au* and *fri* mutants are impaired in the first positive phototropism.

To further characterize the amplification of fist positive phototropism by multiple pulses of blue light the dark-grown wild type and mutant seedlings were treated to five multiple pulses of blue light separated by dark interval of 10 min between each pulse treatment. Figs 4.17 and 4.18 show that wild type seedlings of genotypes AC and MM respond with mean curvature of 47.31° and 38.89° respectively. In contrast, *au* and *fri* mutants responded with mean curvature of 17.90° and 6.88° respectively. (Figs. 4.17 and 4.18). There is a significant increase in curvature in the *au* mutant. These results indicate that amplification of first positive phototropism takes place in *au* mutant compared to *fri* mutant, however, it is insignificant with respect to its wild type in the same background.

4.14 PHOTOTROPISM IN *hp-1* MUTANT

Since *hp-1* mutation can overcome the phyA deficiency in *au* and *fri* mutants, the fluence response curve for blue-light mediated phototropism was determined for dark-grown wild type and *hp-1* mutant seedlings. Seedlings were exposed to a fixed fluence of 0.1 $\mu mol\ m^{-2}\ sec^{-1}$ blue light for various duration and curvature was measured 120 min after the onset of blue light exposure. Fig. 4.19 shows that though the first positive curvatures show a bell shaped profile for both wild type and *hp-1* mutant, the mutant showed higher magnitude of curvatures. The kinetics differed with respect to time threshold needed for *hp-1* to induce second positive curvatures (Fig. 4.20). A clear zone of indifferent curvatures between 120 and 1000 sec i.e., between first and second positive curvature is discernible for wild type, whereas in *hp-1* mutant

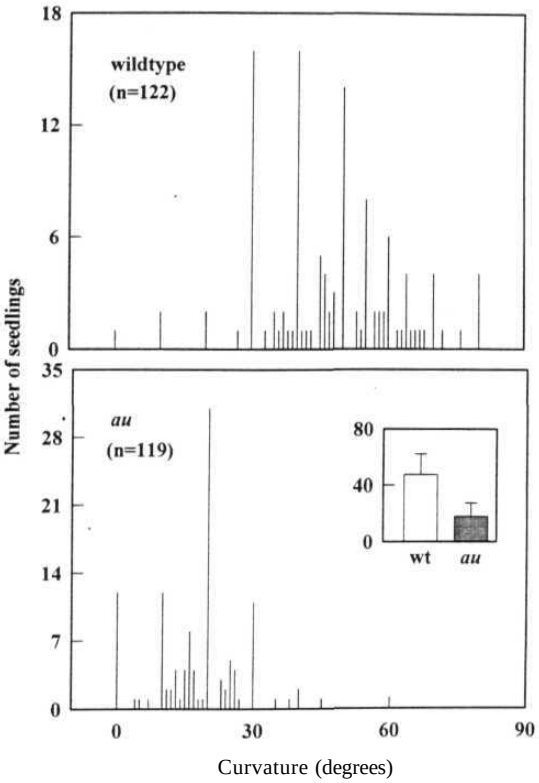

Figure 4.17. Frequency distribution of phototropism in wild type and *au* mutant seedlings to multiple pulses of blue light. The 3-day-old dark-grown seedlings were exposed to five pulses of blue light (450 nm) at fluence 0.1 μmol m^{-2} s^{-1} for 10 sec with 10-min dark interval between each pulse treatment and returned to dark. Curvatures induced were measured 2 h after the first pulse treatment. The inset shows comparison of mean value (± S.D) of wild type and mutant.

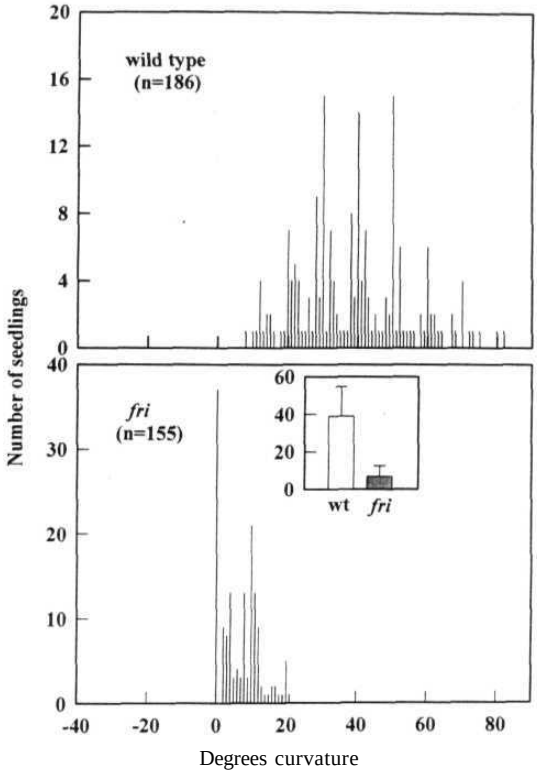

Figure 4.18. Frequency distribution for phototropism in wild type and *fri* mutant seedlings to multiple pulses of blue light. The 3-day-old dark-grown seedlings were exposed to five pulses of blue light (450 nm) at fluence 0.1 $\mu mol\ m^{-2}\ s^{-1}$ for 10 sec with 10-min dark interval between each pulse treatment and returned to dark. Curvatures induced were measured 2 h after the first pulse treatment. The inset shows mean curvature value (± S.D) of wild type and mutant.

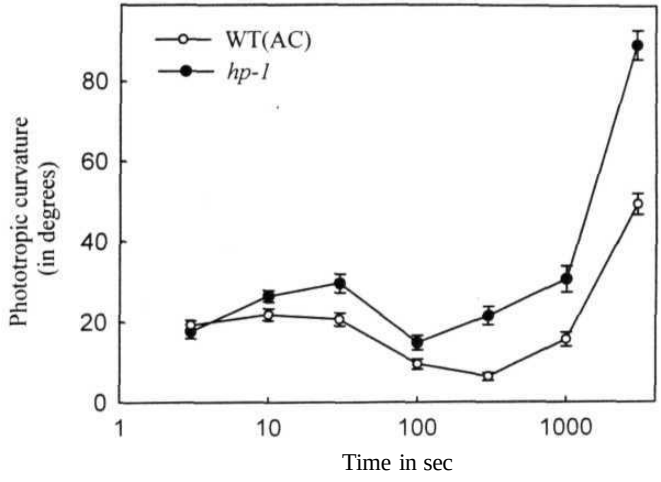

Figure 4.19. Dose-response curve of wild type and *hp-1* mutant seedlings for blue light induced first and second positive phototropism of 4-day old, dark grown seedlings. Curvature was induced by blue light of fluence 0.1 μmol $m^{-} s^{-}$. Each point represents the average of 30 plants. Error bars represent S.E of mean.

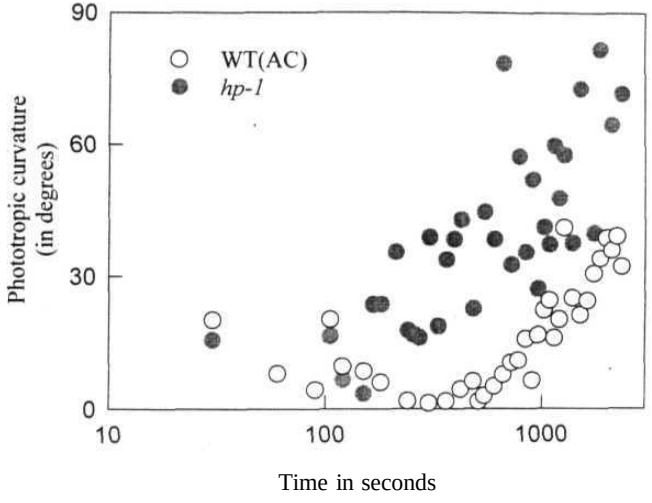

Figure 4.20. Comparison of indifferent zone for blue light-induced curvature in wild type and *hp-1* mutant seedlings. Blue light at fluence 0.1 μ mol $m^{-2}s^{-1}$was administered for different time intervals and incubated in dark. Curvature was determined 90 min after the onset of irradiation. Each datum point shows mean curvature obtained from 10 seedlings.

this indifferent zone was found to be minimal. While time threshold needed for stimulation in WT was 10 min, in *hp-1* mutant it was about 3-5 min. A comparison of phototropic response after 10 min blue light pulses clearly highlights this difference between wild type and *hp-1* mutant (Fig. 4.21) where WT showed a mean curvature of only 7° while *hp-1* mutant showed a significant curvature of about 47°. Similarly the level of second positive response attained in the *hp-1* mutant after 40 min was much higher than the wild type (Fig. 4.20). It is apparent from the above results that *hp-1* mutant shows higher degrees of curvatures to blue light compared to wild type.

4.15 ENHANCEMENT OF FIRST POSITIVE PHOTOTROPISM IN PHOTOMORPHOGENIC MUTANTS

To examine the role of phytochrome in enhancement of phototropic response effect of the different pretreatment to R or FR light were studied in the photomorphogenic mutants *an, fri, tri* and double mutants *tri,fri, fri,hp-1* and *au,hp-1*. The dark-grown or red/far-red light pretreated seedlings of wild type and mutants were exposed to unilateral blue light for 10 sec and curvature was determined after 90 min incubation in dark. From Figs. 4.22 and 4.23 it can be seen that the phyA deficient *an* and *fri* mutants seedlings pre-irradiated with red light or far-red light showed some enhanced phototropic curvature compared to control seedlings. However, in mutant seedlings the responses were dramatically impaired compared to respective wild type cultivars in the same background. By contrast, phyB1-deficient ***tri*** seedlings with or without red light pre-irradiation showed response similar to wild-type seedlings (Fig. 4.23). However, the *trifri* double mutant exhibited reduced phototropic curvature under both irradiation conditions like that observed for phyA single mutants irradiated with blue light alone. Interestingly, the dark grown

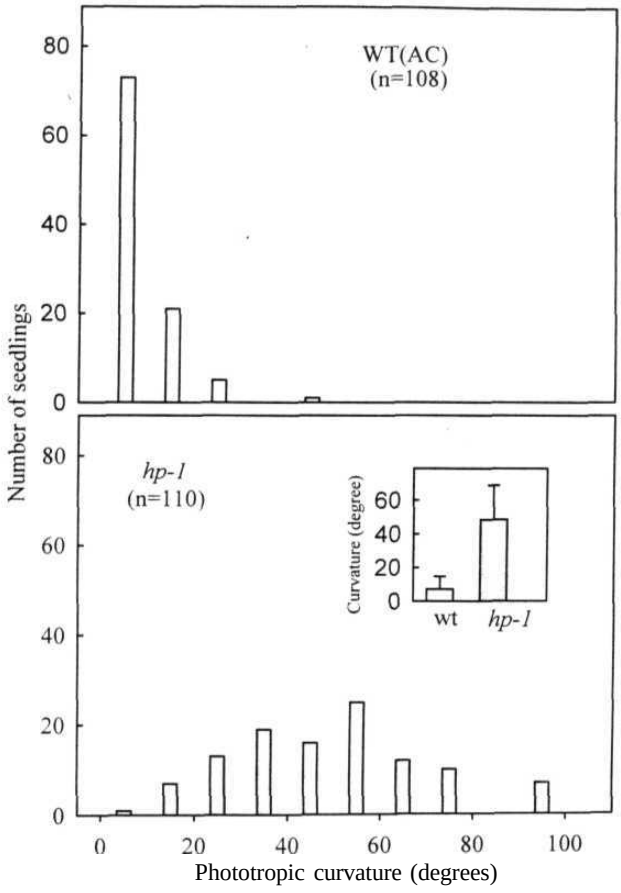

Figure 4.21. Frequency distribution for phototropism of wild type and *hp-1* mutant seedlings to 10 min of blue light. Dark grown seedlings were treated to unilateral blue light at fluence 0.1 μmol m^{-2} s^{-1} and returned to dark followed by measurement of curvature at 90 min interval. In the inset is shown mean comparison of wild type and mutant. Error bars represent S.D of mean.

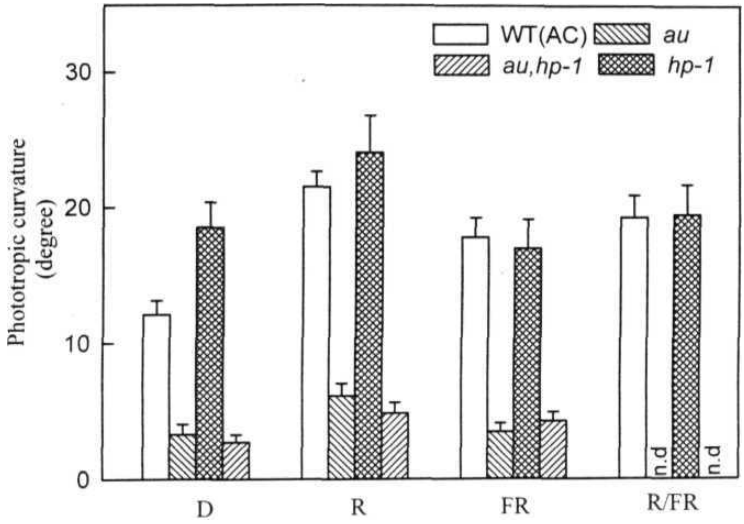

Figure 4.22. Comparison of the enhancement of first positive phototropism in wild type and mutant seedlings. Dark-grown seedlings were pretreated to 5 min R, or 5 min FR, or 5min R followed by FR and returned to darkness for 90 min before exposure to a blue light pulse for 10 sec duration. Curvature was measured at 90 min after beginning of blue light exposure. The means obtained from 30 plants are shown with representative ±S.E values.

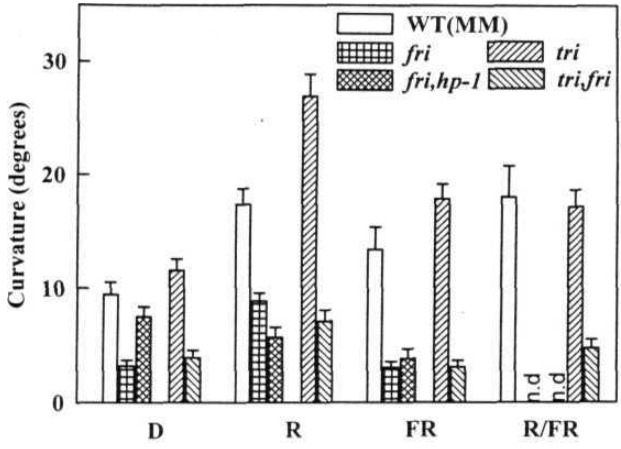

Figure 4.23. Comparison of the enhancement of first positive phototropism in wild type and mutant seedlings. Dark-grown seedlings were pretreated to 5 min R, or 5 min FR, or 5min R followed by FR and returned to darkness for 90 min before exposure to a blue light pulse for 10 sec duration. Curvature was measured at 90 min after beginning of blue light exposure. The means obtained from 30 plants are shown with representative ±S.E values.

hypocotyls of double mutants *au,hp-1* and *fri,hp-1* though normal in second-positive phototropism (similar to *hp-1*), lost the first positive response and are similar to *au* and *fri* mutants respectively (Figs. 4.22 and 4.23).

4.16 ENHANCEMENT OF SECOND POSITIVE CURVATURE IN WILD TYPE AND *hp-1* MUTANT

Stimulation of hypocotyls with unilateral blue light for periods that exceed a few minutes leads to phototropic responses that is strongly dependent on time and pretreatment with R. Figure 4.24 shows time threshold in dark grown and R or FR or R/FR pretreated seedlings of wild type and *hp-1* mutant exposed to blue light for varying duration of time. It is evident from results obtained that second positive response in dark-grown wild type seedlings became apparent after 10 min, and showed a linear increase during next 30 min. By contrast the second positive curvatures are increased substantially with R or FR or R/FR pre-irradiation. However, although the dark-grown seedlings of *hp-1* mutant showed higher degrees of curvature than wild type when compared for same duration, pretreatment with R or FR light had no significant effect on the response.

4.17 PHOTOTROPISM IN DE-ETIOLATED SEEDLINGS OF PHOMORPHOGENIC MUTANTS

Since dark grown seedlings of *au*, *fri* and *tri,fri* mutants are impaired in blue-light mediated phototropism they were tested for phototropism after de-etiolation under white light. The seeds of wild type and mutants after germination were de-etiolated for 24 or 48 or 72 hrs in white light and then examined for phototropic response after exposure to continuous unilateral white light. Fig. 4.25 shows that the mutants show normal phototropic

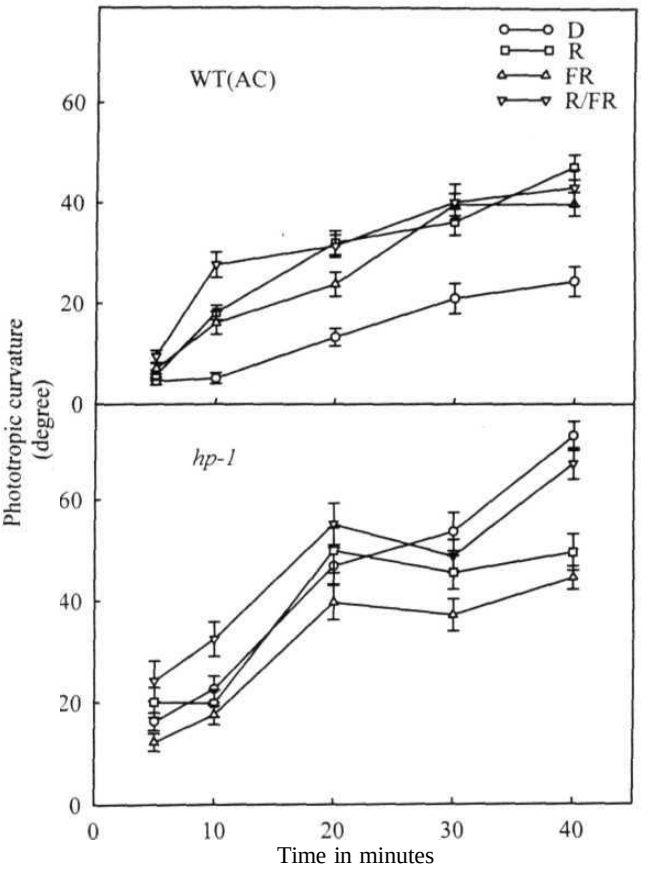

Figure 4.24. Enhancement of second positive phototropism in wild type and *hp-1* mutant seedlings. The dark grown seedlings were omnilaterally irradiated for 5 min with RL or FR. After a dark interval of 90 min, seedlings were stimulated for various duration of time interval with unilateral blue light (450 nm) at a fluence of 0.1 μmol m^{-2} s'. Each point is the mean of 30 seedlings and error bars represent S.E of mean.

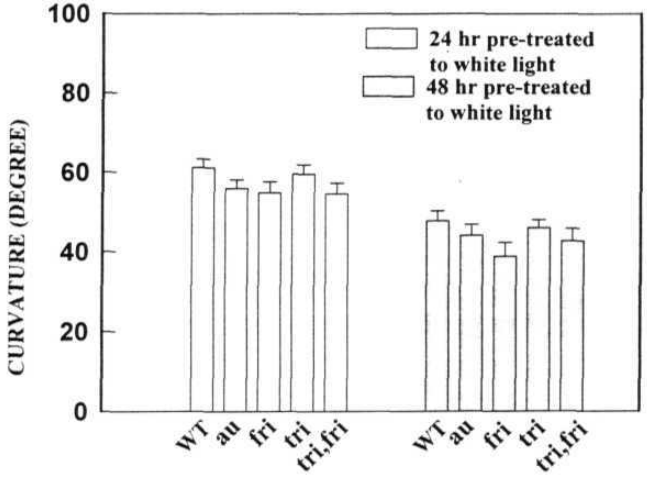

Figure 4.25. Phototropism in wild type and mutant seedlings. Seedlings were grown for 24 hrs or 48 hrs under white light and returned to darkness for 2 hrs. Thereafter the seedlings were stimulated with unilateral white light for 8-12 hrs and curvatures were determined. Each point is the mean of 25-30 plants. The error bars represent S.E of mean.

curvature similar to wild type indicating that the mutants are normal in de-etiolated seedling phototropism.

4.18 ISOLATION AND SCREENING OF NON-PHOTOTROPIC MUTANT LINES

Although Darwin (1890) first documented phototropism more than century ago, little is known about the genetic events regulating this important response. Though some aspects of genetic regulation of phototropism are now known in *Arabidopsis* (Liscum and Briggs, 1995), no mutants were isolated or described in literature in the tomato. In addition, in comparison to *Arabidopsis*, studying phototropism in tomato has some advantages; for example its large seedling size makes it useful for photophysiological analysis. The non-phototropic mutants will also be specifically useful for further elucidation of the mechanism of response and their role in plant growth and development. Hence, an attempt was made to isolate and characterize tomato mutants impaired in phototropism to develop as another genetic model for the study of phototropism.

Seeds were mutagenized with 60 mM EMS (Koornnef et al.,1989) resulting in the M_1 generation of seeds. The appearance of somatic sectors and other morphological variations on M_1 plants were taken as indication of the effectiveness of mutagen (Fig. 4.26). To identify the mutants that are non-phototropic, M_2 seed harvested and bulked from 1500 M_1 plants were screened. The procedure followed is outlined in Fig. 4.27. The M_2 seeds were sown and allowed to germinate in soilrite mixture filled at a density of approximately 200 - 300 seeds per petri-dish and grown in dark for a week. After which the seedlings were then treated to unidirectional continuous blue light, and were scored for seedlings that appeared non-phototropic. From a total of approx.

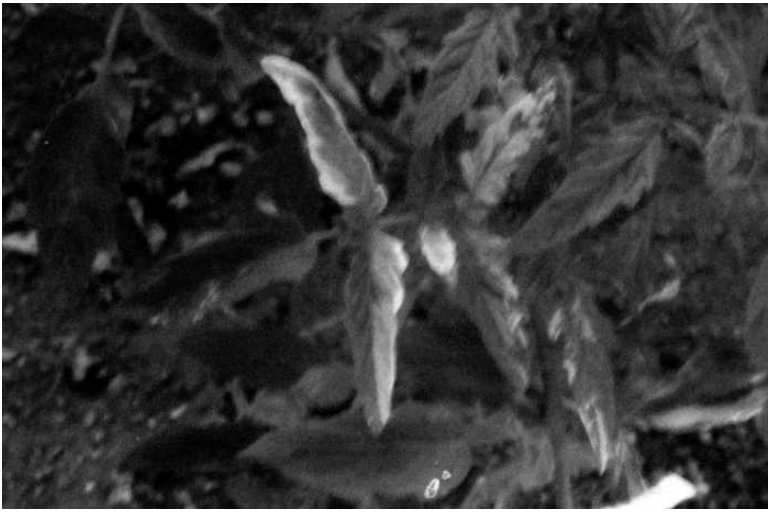

Figure 4.26 Mutant somatic sectors induced by ethylmethane suflonate in M_1 plants.

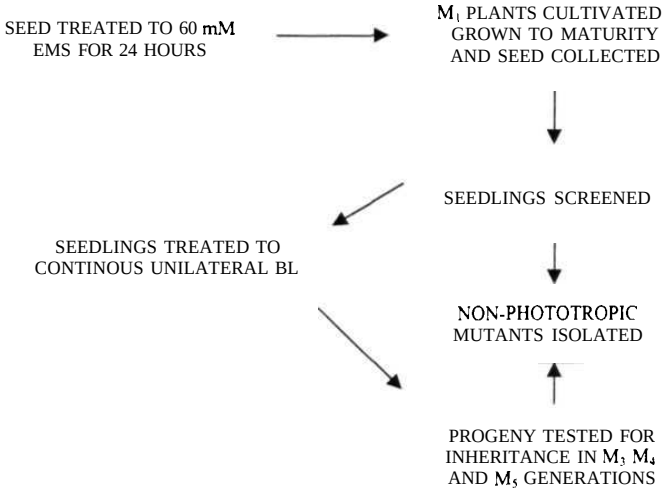

Figure 4.27 Procedure followed for isolation of non-phototropic mutants in tomato.

1,40,000 M_2 seedlings screened, 16 putative mutants, that were insensitive towards unidirectional blue light treatment for 12 -16 hrs of exposure were identified (see Fig. 4.28). The plants were allowed to mature and seed (M_3 generation) from these M_2 plants were further secured for the presence of mutant phenotype as summarized in Table 4.3.

During further screening of the mutants in following generations, 8 lines were found to retain mutant phenotype. Seed of two lines 1EMS96 and 4EMS96 showed wild type response in later generations and hence were not used further. One of the line (6EMS96) though set seeds but could not germinate in M_3 generation and similarly some of the lines (12-16EMS96) were lost as these lines failed to set seeds (Table 4.3). However, two of the lines *(viz.,* 2EMS96 and 5EMS96) along with other non-phototropic lines that retained for phenotype were selected and characterized.

4.19 CHARACTERIZATION OF NON-PHOTOTROPIC MUTANTS

The mutant lines were analyzed genetically and physiologically to determine the nature of the mutation and their relationship. The lines 2EMS96 and 5EMS96 were crossed with wild type and observed that F_1 seedlings are non-phototropic in both the cases (Table 4.4). The analysis of segregation ratios showed approximately 1:3 ratio of phototropic to non-phototropic in F_2 seedlings suggesting that these two mutant phenotypes were caused by dominant mutations. In addition, to clarify the genetic relationship of the phenotype, reciprocal crosses were also performed. The results of these crosses are consistent with the interpretation that the phenotypes are due to single dominant mutation in the respective mutants.

Figure 4.28 M_2 seedlings screened for phototropically altered mutant strain. Seedlings were grown for a week in darkness, then unilaterally illuminated for 12-16 hrs from direction labeled with the arrow. The mutant cannot detect light direction.

Table 4.3. List of mutants isolated in the M_2 generation and inheritance of phenotype in latter generations. M_2 seed were sown in petri dishes and subirrigated with water. All seeds were placed in a growth chamber in dark at 25° C for 4 d prior to phototropic stimulus for 12 h or more. At the end of phototropic stimulation seedlings were selected with no bending in hypocotyls. These were then analyzed for phototropism in each generation.

Non-phototropic mutant line isolated in M_2 generation	Inheritance of phenotype in M_3, M_4 and M_5 generations	Phototropism	remarks
1EMS96			Normal phototropic bending in later generations
2EMS96	+Ve	No bending	
3EMS96	+Ve	No bending	
4EMS96			Normal phototropic bending in later generations
5EMS96	+Ve	No bending	
6EMS96			No germination of seed in M_3 generation
7EMS96	+Ve	No bending	
8EMS96	+Ve	No bending	
9EMS96	+Ve	No bending	
10EMS96	+Ve	No bending	
11EMS96	+Ve	No bending	
12EMS96-16EMS96			no fruits or seeds set in M_2 plants

Table 4.4. Genetic analysis of non-phototropic mutant lines *Nps* - 2 and *Nps* -5.

Type of cross	Phototropism in F_1 generation seedlings		Phototropism in F_2 generation seedlings		ratio	χ^2 test
	Showing response	No Response	Showing response	No Response		
WT X *Nps*–2 (9) (♂)	-	28	81	214	1:2.6	$\chi^2 = 0.948$ 0.5>p<0.3
WT X *Nps*-2 (♂) (?)	--	19	64	180	1:2.8	$\chi^2 = 0.204$ 0.7>p<0.5
WT X *Nps*-5 (♂) (?)	-	17	79	196	1:2.4	$\chi^2 = 1.835$ 0.2>p<0.1
WTXNps-5 (?) (♂)	-	8	92	238	1:2.5	$\chi^2 = 1.308$ 0.3>p<0.2

We have named these two genetic loci *Nps-2* (for *N*on *p*hototropic *s*eedling) and *Nps-5* respectively. The two mutants exhibited morphological characteristics normally associated with wild-type seedlings except for phototropic response. Figure 4.29 shows comparison of 4-day-old etiolated and de-etiolated seedlings of wild type, homozygous *Nps-2*, and *Nps-5* mutants treated to same light conditions. It is evident that etiolated and de-etiolated *Nps-2* and *Nps-5* mutant show ho phototropic response compared to wild type.

Table 4.5 shows measurement of hypocotyl length of wild-type and homozygous *Nps-2* and *Nps-5* seedlings during the first week of germination in dark and light conditions. The hypocotyls of mutants show growth rates similar to dark-grown wild-type seedlings. The inhibition of hypocotyl elongation in mutants under white light was also found to be similar to wild type. It is evident from these results that these mutants are dissimilar to *au* and *fri* mutants, which show elongated hypocotyl to this light condition.

The gravitropic response in mutant hypocotyls and roots were nearly similar to wild type (Table 4.5). Interestingly, when the *Nps-2* and *Nps-5* mutant seedlings are treated simultaneously to phototropic stimulus as well as gravitropic stimulus, the mutant seedlings showed only gravitropism i.e., moved away from the light source whereas wild type seedlings showed phototropism. These results strongly indicates that the mutants are specifically impaired in the phototropic transduction pathway. The mutants also showed a normal ethylene induced triple response and auxin induced growth orientation similar to wild type seedlings.

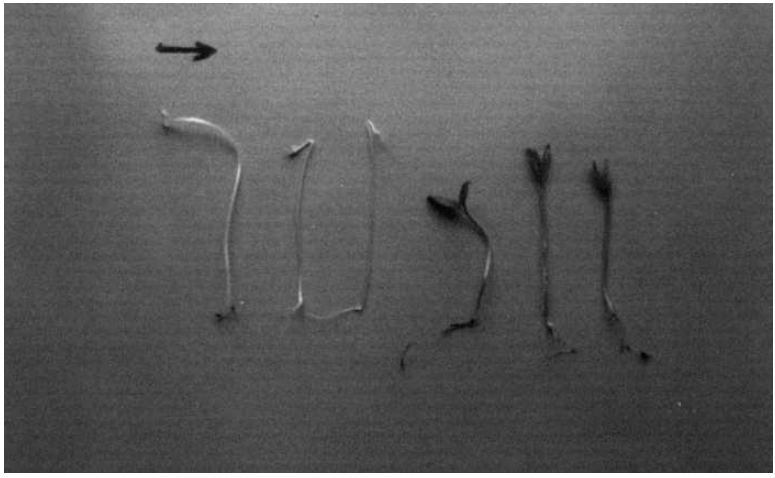

Figure 4.29. Phototropic response of wild type and mutant seedlings germinated in dark or light. From left to right; wild type (etiolated); *Nps-2* and *Nps-5* mutants (etiolated); wild type (de-etiolated); *Nps-2* and *Nps-5* mutants (de-etiolated). The seedlings were illuminated from left (arrow) with continuous blue light (etiolated) or continuous white light (de-etiolated) for 12 hrs.

Table 4.5. Physiological analysis of non-phototropic mutant lines *Nps-2* and *Nps-5*. Hypocotyl lengths were measured from the point of attachment of cotyledons to the base. Following growth of seedlings in dark for 5 day, the average growth rate of hypocotyl was determined. Seedlings were germinated in darkness for 3d in the presence of ethylene and scored for sensitivity. Tod determined auxin sensitivity cotyledons were treated by placing 100 μm auxin solution on the surface of cotyledon. Values are mean ±S.E.

Physiological property studied	WT	*Nps-2*	*Nps-5*
Hypocotyl length (in mm) in de-etiolated seedlings (n=30)	2.2 (±0.07)	2.6 (±0.05)	2.5 (±0.07)
Growth rate in dark grown seedlings (in mm) (n =30)	0.078 mm hr^{-1}	0.078 mm hr^{-1}	0.071 mm hr^{-1}
root gravitropism (in degrees)	37° (±2.5)	29° (±3.1)	26° (±2.4)
hypocotyl gravitropism (in degrees) (n=30)	45° (±2.4)	42° (±2.0)	43° (±2.4)
Simultaneous treatment to gravity and phototropic stimulus (in degrees) (n=30)	- 79° (±2.8)	+ 58° (±3.1)	+ 53° (±2.6)
Triple response (n=15)	+ve	+ve	+ve
Auxin influence on orientation of seedling (n=30)	+ve	+ve	+ve

4.20 SEED GERMINATION IN *au,hp-1*

Although tomato is an example of a D-germinating species, there is evidence that the FR-absorbing form of phytochrome present in seeds is a prerequisite for germination. This was tested in the wild type and *au,hp-1* double mutant which has a phenotype closer to *au* than *hp-1*. Seed of WT were germinated on wet filter paper under total darkness or continuous red or blue or white light and subsequently the germination of each population were measured. Figure 4.30 A. shows a comparison of germination of wild-type under different light conditions. The germination of seed in dark started after two days and reached a maximum after 4 days, thereafter the germination decreased. Compared to the germination of wild type seed in dark, white or red light treatments was found to reduce and inhibit germination. Figure 4.30 B shows comparison of germination in wild type and *au*, *au,hp-1* and *hp-1* mutants. In darkness the *au* and *au,hp-1* mutants showed a reduction in seed germination, however, *hp-1* mutant seed germinated similar to wild type. In contrast, under white or red light conditions *au* and *auhp-1* mutants germinated well. Thus, it appears that phytochrome inhibits germination in red or blue or white light in wild type and the germination response *of au,hp-1* is similar to *au* mutant.

4.21 CHLOROPLAST DEVELOPMENT IN *au* MUTANT

The appearance of proteins of the oxygen-evolving complex, involved in water oxidation in photosynthesis, is known to be controlled by phytochrome and it has been demonstrated that the phytochrome is able to modulate the rate of transcription of the corresponding nuclear genes. To study the effect of deficiency of phytochrome on appearance of these proteins, the chloroplast development was compared in wild type and *au* mutant in a short

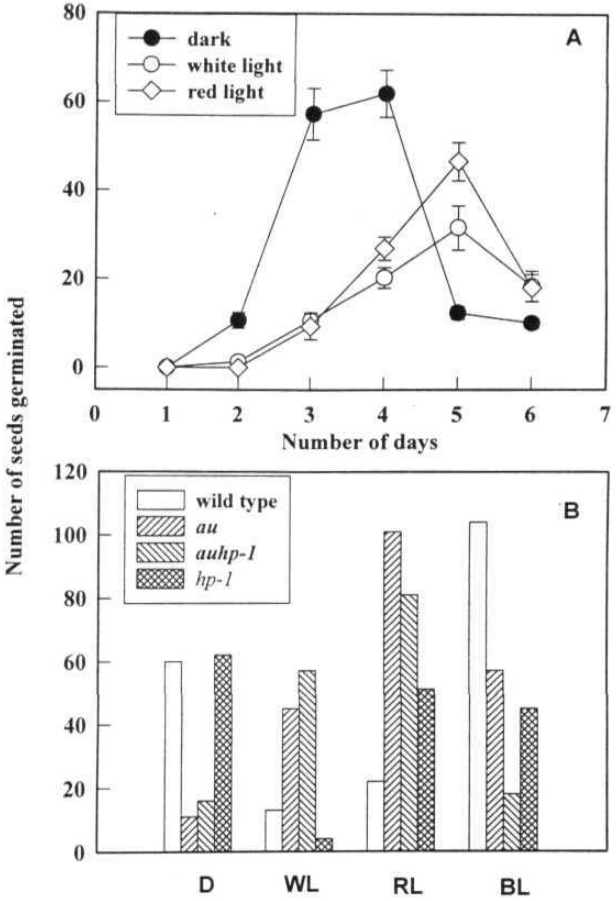

Figure 4.30. Germination of wild type seed with number of days in dark and white or red light conditions (A) and comparison of germination in mutant seed with wild type (B) in dark or white light or red light or blue light of 4 day-old treated seed.

physiological time frame of up to 48 hrs under different light conditions by photoreduction of water oxidizing complex. Wavelength dependence for chloroplast development was monitored by measuring Hill reaction development. **PSI1** activity was measured spectrophotometrically by the photoreduction of DCPIP with water as electron donor. No activity could be detected within the first 8 h of greening for either wild type or mutant thylakoid membranes both in white light and red light (Fig. 4.31). In white light, the rate of development of activity after this lag, however, was faster in the wild type than in the mutant. Although consistently high levels of activities were observed in wild type plastid preparations, both wild type and mutant samples had achieved maximal activities at 48 h of greening. The activities peaked after 40 h and stable activities were not obtained until at least 48 h of greening (Fig. 4.31A).

Compared to this normal development of mutant under white light a different pattern was followed under red light. In red light, photochemical activity of wild type showed steady increase after 8 h and reached a peak at 40 h followed by stable activity at 48 h of greening. In contrast *an* mutant did not develop any photochemical activities after 40 h of greening and with a very insignificant level of activity at 48 h of greening (Fig. 4.31B). These results correlate well with phytochrome function and since *an* is deficient in phytochrome, its chloroplast development is blocked or arrested specifically under red light.

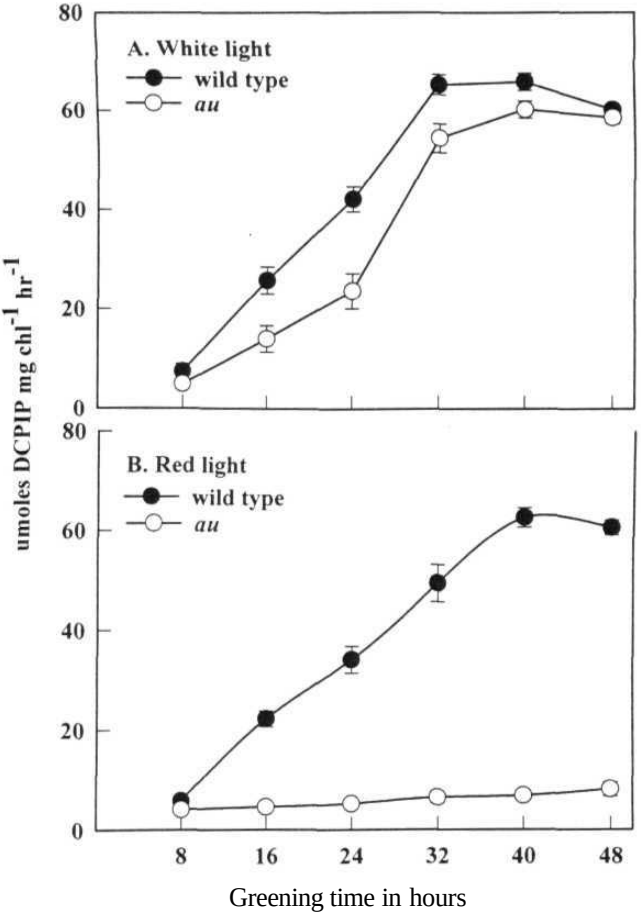

Figure 4.31. Development of photochemical activity ($H_2O \rightarrow DCPIP$) as a functioning of greening in chloroplast preparations from wild type and *au* mutant seedlings grown in white light (A) or red light (B).

CHAPTER 5

DISCUSSION

In recent years, the isolation of mutants and molecular cloning of some of the loci have provided insights into the complex network of light regulated growth and developmental changes involved in the de-etiolation process (Deng and Quail, 1999). Mutants defective in the perception of light signals provided genetic evidence that there are specific multiple types of blue and red light photoreceptors and the red/far-red and blue light signal transduction pathways are genetically separable. ' It has also been well recognized that photomorphogenic seedling development in wild type plants require concerted action of multiple photoreceptors. Thus, these individual pathways are likely to converge downstream upon common regulatory steps that transmit information to the negative and positive effectors inside the cell (Deng and Quail, 1999). The pleotropic phenotype caused by mutation like *hp-1* locus implied that this gene might encode product involved in the light regulatory steps (Peters et al., 1989). To better understand the function of *HP-1* gene product epistatic study between *hp-1* and phytochrome mutants had placed it downstream of phytochrome (Peters et al., 1992b;Kerckhoffs et al., 1997a).

In the present study, we expanded these studies with the mutants to better understand the roles and possibility of interactions between blue/UV-A photoreceptors and phytochromes and to reveal possible hierarchy among phytochromes and the *HP-1* gene in the phototropism of tomato seedlings. Our observations that *hp-1* mutant of tomato is consistent with the *HP-1* gene product function as critical link in multiple signal transduction pathways. Second, the isolation of mutants has been done to find additional elements involved in this signaling pathway. The conclusions drawn from this genetic and physiological analysis provide a framework for further molecular genetic studies towards understanding the mechanism of phototropic signal transduction in tomato seedlings.

5.1 BLUE/UV-A LIGHT INDUCES PHOTOTROPISM IN TOMATO

It is known that unidirectional blue light elicits phototropic growth response in higher plants (Iino, 1990). Likewise in tomato seedlings blue light regulates the phototropic bending toward light source (Fig.4.1). Action spectra for phototropism in *Avena* (Thimann and Curry, 1961) and alfalfa (Baskin and Iino, 1988) show the major peak in blue region, a peak at 370 nm, little action at wavelengths longer than 500 nm, and a peak in UV region at 280 nm. On the basis of such spectra and biochemical (Short and Briggs, 1994) and genetic evidences (Liscum and Briggs, 1995), the responsive pigment has been recently identified in *Arabidopsis* (Huala et al., 1997) and named as phototropin (Christie et al., 1999). The fact that this photoreceptor pigment is associated with phototropic signal transduction in several species including tomato (Short et al., 1993) suggest that blue light induced phototropic response in tomato is most likely mediated by this pigment, but no strong evidence for such relationship between phototropin and the response has yet been produced in tomato seedlings.

5.2 RED LIGHT INEFFECTIVE IN INDUCING PHOTOTROPISM IN TOMATO

The red/far-red absorbing photoreceptor, phytochrome, also absorbs blue and has previously been implicated as a primary photoreceptor for phototropism in dark-adapted maize (Iino et al., 1984a, 1984b; Kunzelmann and Schafer 1985), etiolated pea (Parker et al., 1989) and in selected light-grown monocot and dicot seedlings (Atkins, 1936; Shuttleworth and Black, 1977). However, a 2-hr unilateral red light exposure to etiolated wild-type tomato seedlings did not result in phototropic curvature (Fig.4.1). Similarly, Steinitz et al., (1985) noted that etiolated *Arabidopsis* seedlings exhibited no bending of the hypocotyl when irradiated unilaterally with light at wavelengths at or above 560 nm and at different fluences varying by as much as six orders

of magnitude. Therefore, it appears that the directional light cues that induce phototropic curvature in etiolated tomato seedlings are processed via activation of a putative blue light sensitive photoreceptor exclusively (Liscum and Briggs, 1995). Apparently, blue light-induced phototropism is uncomplicated by phytochrome mediated growth responses.

5.3 BLUE LIGHT INDUCES BOTH FIRST AND SECOND POSITIVE PHOTOTROPISM IN TOMATO

Blue light pulse induced phototropic stimulation evokes two distinct responses: the first positive phototropism characterized by relatively smaller curvatures and second positive phototropism producing larger curvatures (Iino, 1990). The dose-response relationship of phototropic curvature in tomato (Fig.4.2) manifests both these curvatures and several features of this curve are similar to that found in other species. First positive peak curvatures of tomato occur at fluences of approximately 0.1 $\mu mol\ m^{-2}$. In comparison, maximal first positive phototropic curvature of *Avena* is found at approximately 0.1 $\mu mol\ m^{-2}$ (Zimmerman and Briggs, 1963), that of alfalfa, pea, flax and mungbean at approximately 5.0 $\mu mol\ m^{-2}$ (Baskin and Iino, 1987) and that of *Arabidopsis* at 0.5-1 $\mu mol\ m^{-2}$ (Steinitz and Poff, 1986). The major difference between the dose-response curve obtained with coleoptiles of monocot and hypocotyls of dicot seedlings is the zone separating the first and second positive types of response. In *Avena* dose response curves in this zone, negative curvatures are observed with high intensity of light (Zimmerman and Briggs, 1963). However, no such negative curvatures were observed in tomato.

It has been found that the time threshold needed for eliciting second positive phototropism varies with species and range from about 5 min to about 60 min (Blaauw and Jensen 1970; Briggs 1960; Kubo and Mihara 1988;

Zimmerman and Briggs 1963; Janoudi et al., 1992). The data presented here for second positive phototropism is close to values reported for *Arabidopsis* (Janoudi et al., 1992) and is observed at fluences >100 μmol m^{-2} for a time threshold of about 10 min (Fig 4.2). Thus it is evident from the above data that in dark-grown tomato seedlings the blue light photoreceptor system is equally effective in inducing both first and second positive types of phototropic response.

5.4 CAROTENOIDS ARE NOT INVOLVED IN PHOTOTROPIC STIMULUS PERCEPTION BY TOMATO SEEDLINGS

Quinones and Zeiger (1994) recently observed several correlations between the level of zeaxanthin, a carotenoid of the xanthophyll cycle, and phototropic sensitivity in maize. They suggested that zeaxanthin might be the photoreceptor chromophore for phototropism in maize on the basis of coorelation. The data presented in this work indicate that carotenoids are unlikely to be the chromophores mediating phototropism in tomato because treatment with NF did not prevent phototropic response (Fig.4.6). These results, are also consistent with those obtained with oat and maize (Vierstra and Poff, 1981; Palmer *et al.,* 1996) and suggest that carotenoids do not play a role in phototropic response. This is further supported by the observation that the maize coleoptiles devoid of all carotenoids, as a result of either genetic lesion or herbicide treatment, nevertheless showed strong second positive curvature (Palmer et al., 1996). Additionally there is strong evidence that the photoreceptor is associated with the plasma membrane in higher plants (Short and Briggs, 1994; Liscum and Briggs, 1995), not with the chloroplast, where the components of xanthophyll cycle and other carotenoids are located.

5.5 HEAT STRESS INHIBITS PHOTOTROPISM BY GROWTH INHIBITION IN TOMATO

In etiolated seedlings of tomato subjected to heat stress, both phototropism and gravitropism were severely inhibited (Fig. 4.3). The growth response of heat shock treated seedlings is also consistent with this inhibitory action of heat (Fig. 4.4). Despite the well-documented adverse effects of high temperatures (Verling, 1991), the causal mechanism by which phototropism and growth responses are affected is not well understood. Moreover, the sensor for temperature perception has not yet been found. However, several inhibitory processes are known to proceed simultaneously at extreme temperatures (Verling, 1991). While both low molecular weight and high molecular weight heat shock proteins have been estimated but no specific function of these heat shock proteins on response effected is established (Verling, 1991). Moreover, in response to perturbed environmental conditions such as heat, the adaptation shown by many plants could partly be due to changes in membrane composition and phase behavior, which optimized the fluidity (Navari-Izzo et a]., 1993).

It is also proposed that alterations in bulk membrane lipids perturb cell function by inducing changes in the structure and function of several intrinsic membrane protein complexes (Caldwell and Whitman, 1987; Horvath et al., 1989). Murata and Los (1997) emphasized the role of membrane fluidity. They speculate that the sensor is located in microdomains of the membrane and able to detect physical phase transitions which then lead to conformational changes and/or phosphorylation de-phosphorylation cycles due to changes in temperature. In the light of these observations, it appears likely that the inhibition of growth by heat stress might be due to pleotropic effects on cellular membranes. Since it is thought that membranes are site for perception of

phototropic stimulus (Short and Briggs, 1994), perturbation of this structure may result in disruption of phototropic stimulus perception. Alternatively, the reduction in growth might be attributed to disruption of growth regulators or adaptation of plant to the stress condition. The heat stress analysis of phototropism, therefore, can be a useful tool for further understanding the growth changes involved in the response.

5.6 PHYTOCHROME ACTIVATION INCREASES PHOTOTROPIC SENSITIVITY IN TOMATO

In tomato, similar to other plant species when red light, if given alone, is not effective in inducing the phototropism (Fig 4.1). However, it is well known from work with oat, maize coleoptiles and *Arabidopsis* that red light pretreatment prior to a inductive uni-directional blue light pulse, strongly stimulates the manifestation of both first and second positive curvatures (Iino, 1990; Janoudi et al, 1992). The similar observation of stimulation of phototropic sensitivity by red light pretreatment to a subsequent unilateral blue light was obtained in tomato seedlings (Table 4.2 and Fig. 4.7). Janoudi et al., (1992) showed that red light by activating phytochrome leads to the enhancement of phototropism. It can be concluded from the results presented (Table 4.2 and Fig.4.7) that phytochrome enhances phototropism of tomato.

The red/far-red reversibility of phototropic enhancement of first positive curvature could not be detected in tomato seedlings (Table 4.2). This observation is inconsistent with the partial reversibility reported by others studying red light effects on phototropism (Briggs, 1963). Moreover, in tomato FR exposure also enhances phototropic curvature to **a** similar extent to R. It is now known on the basis of physiological and genetic evidences that the perception of red or far-red light is mediated by distinct phytochrome species

(Somers et al., 1991; Dehesh et al., 1993; Mc Cormac et al., 1993; Quail et al., 1995). Phytochrome A, which is abundant in etiolated plants, is probably responsible for very-low-fluence responses which is far-red irreversible (Botto et al., 1996; Casal et al., 1994, 1996; Clough et al., 1995; Mazzella et al., 1997; Shinomura et al., 1996). On the other hand, phytochrome B is responsible for low-fluence responses (Botto et al., 1995; McCormack et al., 1993; Mazzella et al., 1997). The very low fluerice and low fluence responses can be observed even at the level of transcriptional activity of a single gene promoter (Cerdan et al., 1997). It is now believed that mode of action of phytochrome species may be a consequence of combined effect of multiple species on a response and very low fluence and low fluence responses could be from manifestations of different transduction pathways. Therefore, distinct signal transduction pathways by each of participating photoreceptors may be responsible for the enhancement of phototropism.

5.7 PHYTOCHROME ACTIVATION DECREASES TIME THRESHOLD NEEDED FOR SECOND POSITIVE PHOTOTROPISM IN TOMATO

It was observed that seedlings pretreated with red light, operating via phytochrome show time-threshold period required to manifest the second positive curvature compared to dark grown tomato seedlings (Fig.4.7). The pre-irradiation with red light decreases the time- threshold from about 10 min in dark grown seedlings to about 5 min in pre-irradiated seedlings. In contrast in *Arabidopsis* where a comparable study was done time threshold reduced from 15 min to 4 min one red-light pre-irradiation (Janoudi et al., 1992). Similar reduction in time threshold is also observed in other plant species for example de-etiolated seedlings respond more rapidly to a phototropic stimulus than do etiolated seedlings (Everett, 1974; Frasen and Bruinsma, 1981; Hart and MacDonald, 1981; Britz and Galston, 1983). While the mechanism regulating

time threshold is not known at the moment, but in dicot seedlings it has been generalized that the red light pretreated plants are more responsive to phototropic stimuli than the dark grown seedlings.

An increasing body of evidence now indicates that the occurrence of blue-light-dependent phototropism of higher plants is strictly under phytochrome regulation. Since both duration of time threshold and enhancement of magnitude of curvature is regulated by red light (Janoudi et al., 1992) it was suggested that phytochrome may act by modulating the blue light signal transduction pathway. An extreme case of phytochrome regulation of phototropism is maize coleoptiles. In this species manifestation of second positive phototropism mainly needs formation of Pfr before it become responsive to unilateral blue light (Liu and Iino, 1996 a,b). It was suggested by Liu and Iino, (1996b) that the Pfr produced by the red light given before a blue-light pulse brought about the increased responsiveness. The results presented here imply that both the blue/UV-A and phytochrome photosystems are equally effective in tomato seedlings, although the activation of phytochrome is not required for the manifestation of first and second positive phototropism.

5.8 INDEPENDENT AND INTERACTIVE ACTION OF PHOTORECEPTORS IN PHOTOTROPISM OF TOMATO SEEDLINGS

Since the phototropic curvature is induced only by blue light but shows strong enhancement by red light indicating that though the photoreceptors may trigger separate signal transduction chains, there is no evidence for how these two chains may share the common steps. In tomato a pre-irradiation with blue light eliminates the first positive phototropism (Fig.4.8). Interestingly, the effect of red or far-red light pretreatment, which stimulates first positive curvature, is drastically reduced when seedlings were first irradiated with blue

light immediately after a R or FR exposures. By contrast if blue light irradiation followed with red or far-red light exposure restores first positive phototropism in blue light desensitized seedlings. It is likely that blue light mediated inhibition of first positive response might be an effect at the level of the photoreceptor. However, there is no evidence for this but CRY1 and CRY2 blue/UV-A photoreceptor are known to decline with blue light irradiation (Ahmad et al., 1998a). On the other hand the red or far-red light may mediate the sensitization of phototropic response (Fig.4.8) perhaps on a later stages of the signal response chain.

Analysis of the interaction between blue and red light by simultaneous treatment with white light and sensitization with red or far-red light demonstrated that first positive response is not restored indicating that branching between blue light mediated phototropic and red light stimulation might not be possible (Fig. 4.8). If it were then, results would be obtained similar to blue light desensitized seedlings with red or far-red light. Previous work on the contributions of blue light photoreceptor and phytochrome to the photocontrol of hypocotyl elongation in de-etiolated *Cucumis* ***sativus*** (Gaba and Black, 1979) has been described as a 'summative interaction' between the two pigments (Mohr, 1994). The present work, shows that under white light, blue light photoreceptor is ineffective except in the presence of Pfr, suggests, however, that there may be a facultative or even an obligatory role for phytochrome in the phototropic signal transduction pathway. Thus, it seems feasible to assume common transduction of blue and red light mediated effects on phototropism and divergence of response to these wavelengths might be due to different modes of action of phytochrome photoreceptor (s).

5.9 PHOTOTROPIC RESPONSE REDUCES WITH DE-ETIOLATION IN TOMATO SEEDLINGS

While the white light grown seedlings do not show first positive curvature, they only show second positive curvatures (Figs. 4.9 and 4.10). It is likely that first positive response is lost during de-etiolation but the seedlings retain second positive response. The reason for loss of first positive response is not known but can only be speculated. One of the possibilities is that it may need type I phytochrome of phyA which is down regulated by light (Furuya 1993). It has been shown that type I phytochrome disappears in light and this can account for disappearance of first positive response during de-etiolation under white light. However, red light does not appear to be responsible for this effect (Fig. 4.9) indicating that the type II phytochromes, which are light stable, might be involved in the enhancement of first positive response and retainment of second positive responsive system during early stages of de-etiolation.

5.10 GENETIC EVIDENCE THAT PHYA IS ESSENTIAL FOR SECOND POSITIVE PHOTOTROPISM IN TOMATO SEEDLINGS

It is evident from the above results obtained in tomato that the induction of a phytochrome-activated signal modifies the signal transduction initiated through the activation of the blue light photoreceptor for phototropism. In the absence of clear evidence of co-action between blue-light photoreceptor and phytochromes, most recent studies have relied on studies of red/blue light initiated responses in the mutants defective in specific photoreceptor species. The studies on phytochrome mutants have been useful in establishing the function(s) to the individual members of the phytochrome family and understanding the mechanism(s) of co-action with blue light photoreceptor in phototropism (Parks et al., 1996; Janoudi et al., 1997a,b). To understand how phytochrome modulates the phototropism, we studied the blue light mediated

first and second positive phototropism in *au* , *fri* and ***tri*** mutants that are phytochrome deficient, in order to better understand the reaction pathway of phototropic response in tomato hypocotyls.

Results presented in Fig. 4.11 show that second positive phototropism in the *au* differs substantially from that of its wild type parent. The *au* mutant requires longer duration of blue light treatment to induce the response (Fig. 4.11). The lag phase for second positive phototropism in the *au* mutant is approximately 3 hr which is about two fold longer than that exhibited by the wild type parent (Fig. 4.11). This observation suggests that phytochrome(s) have an important role in determining the induction of second positive phototropism in tomato. Furthermore the *au* mutant is also impaired to phototropism by white and UV-A light indicating that phytochrome is required for co-action and can also modulate phototropic response to these wavelengths (Fig. 4.12).

The behavior of the *au* mutant is in general, consistent with its low (light labile) phytochrome levels. However, the chromosomal location of the *au* mutation does not appear to correspond with the tomato phyA gene (Sharrock et al., 1988). One of the known effects of the *au* mutation is the reduced type I phytochrome levels (Koornneef et al., 1985; Parks et al., 1987; Lopez-Juez et al., 1990). The *au* mutant has reduced levels of spectrometrically detectable phytochrome and is strongly deficient in the PHYA protein (Terry and Kendrick, 1996; Sharma et al., 1993). Analyses of light-dependent inhibition of hypocotyl elongation and anthocyanin synthesis have demonstrated that *au* seedlings are deficient in both phyA and phyB1 activities (Koornneef et al., 1985; van Tuinen et al., 1995a, 1995b; Kerckhoffs et al., 1997a,b). It is likely that decrease in phototropic sensitivity of *au* mutant could be the result of its general phytochrome deficiency. Alternatively, the *au* mutation may cause

pleotropic effects leading to reduction of phytochrome apoprotein, but affect the BL/UV phototropism and/or the signal transduction chain originating from it as well. Hence, it appears that the labile pool of phytochrome is involved in inducing the second positive phototropism during the first 3 h of de-etiolation.

It is interesting that the *au* mutant does respond after 3 h of phototropic stimulation. It has been shown that light-grown *au* plants retain phytochrome responses and photoactive light-stable phytochrome can be detected in light-grown plants (Sharma et al., 1993). Obviously the low level of phytochrome still detectable in *au* mutant might establish Pfr for a detection of response or that stable phytochrome pool (type II) which is active in white light grown seedlings might be involved in inducing the response. Therefore, *au* mutation can be considered as a leaky mutant for phototropic response.

Other photoresponses, which are strongly regulated by BL/UV-A photoreceptors in addition to phytochrome, have been investigated in *au* mutant. The *au* mutant does not produce any detectable anthocyanin upon red light and blue light treatment (Adamse et al., 1989) whereas in wild type seedlings it is predominantly under the control of BL/UV-A light photoreceptor, while phytochrome (Pfr) alone is not very effective thus indicating the activation of both receptors. Furthermore, the *au* mutant shows reduced photosensitivity to hypocotyl elongation inhibition in blue light and UV-A (Adamse et al., 1988), level of cab PSII transcript after blue light treatment (Oelmuller et al., 1989). The phototropic response of *au* mutant of tomato indicate that a close interaction between blue/UV-A photoreceptors and phytochromes takes place in blue light mediated responses.

Further support for the role of phytochrome(s) in phototropism comes from study of phototropic response of phy A deficient mutant, *fri*. The insensitivity to unilateral blue light observed in/n indicates that phytochrome A is essential for manifestation of second positive phototropism (Fig. 4.13). This observation indicate a strong interaction for blue/UV-A photoreceptor and phytochrome A in phototropism of tomato. Although it has been established that phyA is the dominant or sole regulator of de-etiolation under FR-HIR conditions (Quail et al., 1995), the present study indicates that phy A action is not limited to FR-HIR and it could be responsible for VLF response like phototropism. Similar evidences for role of phyA in VLF is presented for seed germination of *Arabidopsis* (Shinomura et al., 1996) and CAB gene expression induction (Hamazato et al., 1997).

The results obtained with *au* and *fri* mutants of tomato, which show either increased lag phase for second positive phototropism or complete loss of response, are very different from observation made with phyA-deficient mutant in *Arabidopsis* (Janoudi et al., 1997). Apparently, it appears that phyA has a different function in tomato compared to *Arabidopsis.* Characteristically, one feature distinguishes *au* and *fri* mutants from phy-deficient mutants of *Arabidopsis:* in these species, phyA-deficient mutants have phototropic response similar to wild type, but phyA,phyB double mutants show a 6-fold increase in duration of time-threshold (Jaoundi et al., 1997). The extension of time threshold duration indicates in *Arabidopsis* both the phytochromes contribute to the phototropic response in blue light. However, after 2 hr seedlings do show curvature, whereas in/n mutant of tomato seedlings second positive is lost completely. It is possible that the existence of redundancy between phyA and phyB in *Arabidopsis* might have been responsible for the differences observed.

5.11 GENETIC EVIDENCE THAT PHYA IS ESSENTIAL FOR FIRST POSITIVE PHOTOTROPISM IN TOMATO SEEDLINGS

It is assumed that the weak bending response traditionally characterized as first positive phototropic curvature may have a rate-limiting step in the phototropic signaling pathway and this step is somehow influenced by phytochrome photoconversion. Such a influence of phytochrome on overcoming rate-limiting step in the development of phototropism has been proposed in *Arabidopsis* (Steinitz and Poff, 1986; Janoudi et al., 1992), oat (Steinitz et al., 1988), maize (Iino, 1987) and Sesame (Wortzik and Mohr, 1988). Moreover, the examination of relative role of different phytochrome species using mutants in *Arabidopsis* hypocotyls indicated that phototropism in phyA-101 and phyB mutants show a normal first positive, whereas in phyA/phyB double mutant magnitude of the curvature is reduced suggesting that both phytochromes control first positive response in *Arabidopsis* (Janoudi et al., 1997). By contrast, in tomato the altered first positive phototropism exhibited by the *au* and *fri* mutants (Figs. 4.15 and 4.16) results from a deficiency in functional phyA and, therefore, that phyA is the predominant phytochrome mediating blue-light-induced first positive phototropic response in tomato.

In tomato, sequential exposure to five brief blue light pulses separated by relatively long dark intervals very effectively stimulated phototropic curvatures in wild type seedlings (Fig. 4.17). Using the sequential blue light pulses, curvatures with magnitude nearly similar to second positive response can be induced by stimuli with characteristics usually associated with first positive signals. A comparison of the effects of sequence of pulses in wild type and mutants seedlings show considerable differences (Figs. 4.17 and 4.18). The increased curvature obtained in *au* mutant could be due to the intervening dark

periods, which would permit regeneration of the sensitive light receptor and consequent maximally efficient use of the next pulse. A second explanation, complementing the first, is that the earlier pulses may sensitize the photoreceptor system, causing it to respond more effectively to the later pulses. It has been proposed that both light-dependent regeneration of a light-sensitive receptor and a light-dependent increase in the responsiveness of the receptor are necessary to explain the differences in response to single and to pulse stimuli (Steinitz and Poff, 1986). These data further support the hypotheses (Stenitiz and Poff, 1986) that first and second positive curvatures share many features and that a single common mechanism, involving both a dark regeneration of photoreceptor sensitivity and a photoinduced increase in responsiveness, may operate in both systems. The stimulation of responsiveness following a preceding pulse appears to be a common property of blue light responses of plants, as demonstrated in the blue light-induced stomatal response in *Commelina* (Iino et al., 1985) and blue light-induced cell division in *Adiantum protonemata* (Iino et al., 1988) in which after stimulation with a saturating pulse, responsiveness to another pulse was gradually restored.

5.12 PHYA AS BLUE-LIGHT PHOTORECEPTOR OF PHOTOTROPISM IN TOMATO SEEDLINGS

Although phytochrome is capable of absorbing and responding to blue light (Kendrick and Kronenberg, 1994), action spectrum clearly show that blue-light-induced phototropism is controlled by a separate, unrelated sensing system (Liscum and Briggs, 1995). However, the results obtained in tomato clearly shows the dependence of blue light photoreceptor on phytochrome A. In this case, blue light might have also activated phytochrome, which in turn stimulated phototropism. The genetic evidences obtained from phytochrome deficient mutants indicate that phytochrome can also act as blue light receptor

in different responses. Under continuous BL, phytochrome deficient mutants show virtually wild-type responses suggesting that phyA and BL photoreceptors act independently in an additive manner and contributes to the blue light induced hypocotyl growth inhibition response (Koornneef *et al.*, 1980; Young et al. 1992; Liscum and Hangarter, 1994). By using phyA, phyB, and phyAphyB double mutants of *Arabidopsis*,it has been shown that PHYA is the most sensitive blue light receptor for the induction of seed germination (Shinomura *et ai,* 1996) or LHCB gene expression in VLF. Moreover, PHYB and an additional phytochrome of unknown identity contribute to a LF blue light induction of LHCB which shows far-red light reversibility (Hamazato et al., 1997). Similarly, by using double mutants *phyA.phyB* it has been shown that the presence of either phyA or phyB is required for first positive phototropism and time threshold of second positive phototropism (Janoudi *et ai,* 1997; Hangarter, 1997). Furthermore, it has been demonstrated that white and blue light induced accumulation of anthocyanin requires the presence of at least one of the phytochromes: either phyA or phyB (Kunkel *et ai,* 1996; Ahmad and Cashmore, 1997).

5.13 SYNERGISM BETWEEN BLUE/UV-A AND PHYTOCHROME PHOTORECEPTOR IN PHOTOTROPISM

The physiological and genetic studies on phototropism in tomato raise an important question about the mode of interaction between the two photoreceptors. It might be suggested that blue light absorbed by blue/UV-A photoreceptor, could strongly increase the sensitivity of the system towards Pfr. In that case, phytochrome may act on several ways such as, as an antenna pigment, phytochrome as a trap pigment, or it can modulate the amount or quantum efficiency of the blue light photoreceptor pigment. This reasoning would argue in favour of the hypothesis that the phototropic signal-transduction

chain originating from a BL/UV-A photoreceptor would require Pfr for its action (Drum and Mohr, 1984; 1988; Fluhr and Chua 1986; Kendrick and Kronenberg 1994; Mohr, 1994). Nevertheless, it would be premature to conclude the exact role of phytochromes but can be hypothesized that the process of phototropism in tomato seedlings is accomplished with the aid of the phytochrome systems (Liu and Iino 1996a,b).

On the other hand, three possibilities can be considered a) the photoreceptors act separately on differential growth at different sites but this effect interact b) the photoreceptors act separately at the same site on signal transduction chain c) there is direct molecular interaction between the photoreceptors. Our findings indicate that in tomato either of these possibilities is involved to some extent but no definite evidence exists to favor any one of the above possibilities. Some evidence does exist that blue/UV-A photoreceptors, cryptochromes and phytochrome, may interact directly, at least *in vitro* (Ahmad et al., 1998b). However, there is no definite evidence for such an interaction between phototropin and phytochrome A *in vivo.* Irrespective of this open question, the data of the present study seem to be compatible with the concept advanced previously to explain the spectral dependence of light-mediated anthocyanin synthesis (Mohr and Drumm-Herrel, 1983) that the only effect of blue light is to establish and to maintain responsivity to phytochrome.

Parker et al., (1989) interpreted the phototropic response of totally etiolated pea epicotyls to short blue light pulses on the basis of phytochrome action. Based on the previous results with dark-adapted maize mesocotyls (Iino et al., 1984a,b; Kunzelmann and Schafer, 1985) they proposed that epicotyl curvature in their experimental conditions was induced by Pfr gradient established across the epicotyls after illumination with blue light. A related

mechanism may also account for the phototropism induced by continuous blue light in our experiments. Though, Shorpshire and Mohr (1970) were able to demonstrate the existence of a light gradient in red and far red light across the hypocotyl tissue of etiolated seedlings of *Sinapis* and *Fagopyum*. An unilateral red irradiation alone fails to trigger a phototropic reaction of coleoptiles and hypocotyls, even though their growth is often phytochrome-mediated (Gaba and Black, 1983).

5.14 PHYB1 DISPENSABLE FOR FIRST AND SECOND POSITIVE PHOTOTROPISM IN TOMATO

In contrast to phytochrome deficient *au* and *fri* mutants, *tri* seedlings respond to first and second positive phototropism similar to wild-type (Fig 4.14) indicating that phy B1 is largely dispensable for tomato seedling phototropism under these conditions. This indicates that overlapping function of phyB1 and phyB2, phyE, and phyF might be possible in phototropism of tomato seedlings. This is consistent with numerous reports showing that several phytochromes converge to control various processes from the level of gene expression (Reed et al., 1994; Carabelli et al., 1996; Hamazato et al., 1997) to morphogenesis (Halliday et al., 1994; Reed et al., 1994; Develin et al., 1996; Aukerman et al., 1997; Weller et al., 2000). However, one should not rule out that similar genes may contribute in a quantitative different way in different species, as has been shown by the comparison of phytochrome B-type mutants in tomato and *Arabidopsis* (Van Tuinen et al., 1995b).

5.15 PHY A AND OTHER PHYTOCHROMES REQUIRED FOR ENHANCEMENT OF PHOTOTROPISM IN TOMATO

The importance of the phytochrome photoreceptor family in the control of enhancement of phototropism is well established. Usually the red light pre-

irradiated seedlings exhibit an exaggerated phototropic curvature in response to unilateral blue light (Chon and Briggs, 1966; Janoudi and Poff 1992). Despite highly impaired phototropic response during long-term irradiation with blue light, the *au* and *fri* mutants exhibited an approximate 1 fold increase in phototropic curvature when preirradiated with red light (Figs. 4.22, 4.23). The fact that *au* mutant is more or less blind to red light and proved to be deficient in biosynthesis of chromophore (Terry and Kendrick 1996), thus deficient in all types of phytochromes, gives an additional indication of the involvement of phy B and /or other phytochromes in the enhancement process. It appears that this red light-dependent enhancement of phototropic curvature requires phyB phototransformation primarily, if not exclusively, since *tri,fri* double mutant seedlings had phototropic curvature similar to *au* and *fri* seedlings irradiated with blue light alone (Figs 4.22, 4.23). Thus, although phyA appears to be required for the development of normal second positive curvature phyB can apparently provide partial redundant function in the absence of phyA. However, this redundant function is only apparent when seedling were exposed to both red and blue light. Alternatively, the results presented indicate that the responses shown in phyB1 mutant may be entirely attributable to the action of phyA.

It may be worth relating results obtained in tomato with those obtained for *Arabidopsis* mutants. The findings of present study appear to support those of a previous study by Parks et al., (1996) in *Arabidopsis*, where no enhancement of first positive phototropism was observed in the absence of phyA. Furthermore, detailed study on phyA and phyB mutants and transgenic lines overexpressing these phytochrome species, Janoudi et al., (1997a,b) indicated that the involvement of phyA is essential in the very-low- to low-fluence range for enhancement. Whereas either phyA or phyB is required for the high-fluence

enhancement by red light. However, the mechanism by which phytochrome enhances phototropic curvature is largely unknown. Previous studies have shown that enhancement is maximized when the red-light stimulus precedes the blue light phototropic stimulus by 2 hr (Janoudi and Poff, 1991), and it was suggested that phytochrome effects the phototropic response by modulating a component of the blue-light signal transduction sequence (Janoudi and Poff, 1992). Taken, the results from studies of phototropism in *au* and *fri* mutants indicate that the establishment of a large phototropic curvatures occur primarily through a PfrA-dependent enhancement of a limited phototropic response that is initiated via a blue light photoreceptor. Furthermore, these phytochrome-dependent enhancement of phototropism apparently result from positive regulating roles of phyA and phyB on a step(s) either in the blue/UV-A activated signal/response pathway or independent of the blue light photoreceptor derived signals.

5.16 *HP-1* IS A NEGATIVE MODULATOR OF PHOTOTROPISM IN TOMATO

The *hp-1* is a pleotropic mutant and shows high levels of anthocyanin, reduced height of light-grown seedlings (Peter et al., 1992a; Kerchoffs et al.,1997a) and increased flavonoid accumulation in ripe fruits (Yen et al., 1997). Furthermore, the photoinduction of several enzymes in biochemical pathways: PAL (Goud et al., 1991), nitrate reducatase, nitrite reductase, and amylase (Goud and Sharma, 1994), are amplified in the *hp-1* mutant compared with WT. Similarly the *hp-1* mutant showed higher degrees of curvatures of first and second positive response than wild type (Fig. 4.19). The exaggerated features of de-etiolation in *hp-1* mutant were previously shown to be phytochrome regulated, and therefore, it can be concluded that the *hp-1* mutant shows higher magnitude of phototropic response due to phytochrome amplification.

The physiological evidences in *Arabidopsis* suggest that red light pre-irradiated seedlings show a decrease in time threshold for second positive phototropism indicating a definite role for phytochrome (Janoudi et al., 1991). In contrast, the *hp-1* mutant exhibits a minimum time threshold and higher second positive curvatures in blue light alone (Figs 4.19, 4.20 and **4.21).** Moreover, R/FR irradiation has no effect on time threshold in *hp-1* mutant (Fig. 4.24). This indicates that for reducing the time threshold of second positive phototropism phytochrome activation is necessary to down regulate the level of *HP-1* gene product, which might impede the phototropic signal pathway or alternatively *HP-1* might convey hypersensitivity to phytochrome.

The *fri,hp-1* double mutant shows second positive phototropic response similar to wild type, demonstrating that the *fri* mutation is not completely epistatic to *hp-1*. Similarly *au,hp-1* double mutant also show second positive phototropic response suggesting partially epistatic nature of *an.* This is corroborated by the evidences that dwarf phenotype, pigmentation in fruit of *hp-1* mutant is also seen in the *au,hp-1* double mutant. The interaction of the *au* and *fri* with *hp-1* in double mutants (Figs 4.11 and 4.13) suggests the operation of phytochrome pathway controlling seedling phototropism and the *hp-1* product is to be down-regulated for phyA-mediated phototropic response. In a formal genetic sense, *HP-1* gene product therefore defines a novel negative modulator in the pathway of phototropic signal transduction from phyA or other phytochromes.

One interpretation of the observation that the *hp-1* restores second positive phototropism in *au,hp-1* and *fri, hp-1* mutants is that function of phyA and phyB1 might be interchangeable or redundant in response to phototropic stimulus. This also suggests that there could be antagonism between phyA and

phyB1, as has been reported for hypocotyl elongation growth in *Arabidopsis* (Johnson et al., 1994). In *Arabidopsis*, where the availability of phytochrome mutants have been used to examine phototropism, both phyA and phyB were shown to play predominant and overlapping roles (Poppe et al., 1996; Robson and Smith, 1996). Similarly the receptors can compensate for one another in the induction of CAB gene expression (Reed et al., 1994) and in regulation of cotyledon expansion, leaf blade size, and internode and petiole length (Devlin et al., 1996). Since the double mutants *au,hp-1* and *fri,hp-1* regains second positive phototropism, the possibility exists that phytochromes other than light labile pool might enhance the response in the absence of *HP-1* gene product. In the *au* and *fri* mutant, FR is still able to photoconvert phytochrome by depletion of the FR-absorbing form of phytochrome (Pfr), as shown in EODFR, LFR and germination experiments in which the mutants resemble WT. These responses are therefore apparently mediated by other phytochromes, whose effectiveness is determined by the photoequilibrium between R-absorbing form of phytochrome (Pfr) and Pfr at any particular wavelength, meaning that these phytochromes might be mediating the phototropism in the double mutants.

Though the results in the present study are consistent with the notion that phytochrome and *hp-1* act in the same pathway to control photoresponses, we cannot completely exclude the possibility that blue light photoreceptor also function in the same pathway. In addition, we do not know whether *hp-1* also acts down stream of blue light photoreceptor. Since the alleles used in the present study are leaky, the study on interaction between them is not foolproof. Based on physiological evidences, the phytochrome responses are envisaged to be under the constraint of the *HP-1* gene product and both B and the *hp-1* mutation appear to be able to relieve this constraint (Peters et al., 1989, 1992b).

On the other hand, phenotype of *hp-1* appears to be identical to those obtained by ecotopic expression of PHYA in tomato (Boylan and Quail, 1989) and *in vivo* spectrophotometric and immunochemical analysis failed to provide evidence that the *hp-1* mutant is a photoreceptor mutant (Peters et al., 1992b; Kerckhoffs et al., 1997a). Conversely, double mutant analysis of *hp-1* with Phy A and PhyB-1 deficient tomato mutants has demonstrated that the *hp-1* mutation can amplify responses mediated by both phytochromes and that the amplification phenotype is critically dependent upon the presence of an active phytochrome (Peters et al., 1992; Kerckhoffs et al., 1997b). It appears, therefore, that the *hp-1* mutation affect fairly specifically the responses mediated by phytochrome.

It is proposed that the *hp-1* mutation is associated with an amplification step in the phytochrome-transduction chain (Peters et al., 1992b; Kerckhoffs et al., 1997a; Kerckhoffs and Kendrick, 1997). Therefore, it appears that promotion of phototropism by phyA is achieved by a reduction in the level of this inhibitor. Furthermore, because *hp-1* is recessive and mimics its action in double mutants due to response amplification, it is imperative to suggest that wild type *HP-1* gene product encodes a negative regulator that is likely to be involved in the pathway by attenuating or terminating the phy A signal. Although the identity of this inhibitor is currently unknown, its effects have been well characterized (Kerckhoffs et al., 1997b), and one other non allele, *hp-2,* is known to be similar to *DET-2* negative regulator of photomorphogenesis in *Arabidopsis* (Mustilli et al., 1999) further supporting the negative regulation by HP-1 in phototropic response of tomato.

5.17 RELATIONSHIP OF PHY A, BL/UV-A PHOTORECEPTOR AND **HP-1** IN PHOTOTROPISM OF TOMATO

Based on the genetic studies, the possible models that depict how light signals perceived by the blue/.UV-A receptor are transduced through a common cascade of regulation steps, defined by *hp-1* mutation, leading to expression of phototropism are shown in Fig. 5.1. In model 1. phyA and blue-UV-A photoreceptors are proposed to act upstream of HP-1 in the same regulating circuitry. Based on their physiological properties of phytochrome are place in the same hierarchical position as blue/UV-A photoreceptor. In another model HP-1 and positive regulators could interact in one of three possible ways 1. positive regulators upstream of HP-1 2. HP-1 upstream of positive regulators or 3. HP-1 and positive regulators in parallel pathways but converging downstream. In this model, light signals are perceived by multiple photoreceptors, transduced by way of specific early steps, and then converge to other inactivate HP-1 and/or active positive regulators to bring about downstream phototropic response.

5.18 EARLY ROLE OF NPS-2 AND NPS-5 MUTATIONS IN PHOTOTROPISM

The use of mutagenesis was chosen as a genetic tool for the identification of the putative non-phototropic mutants. The non-phototropic mutants were isolated specifically on the basis of having a phenotype of lack of phototropic sensitivity to unilateral blue light. Of particular interest were two mutants *Nps-2* and *Nps-5,* which were characterized genetically. When the homozygous *Nps-2* or *Nps-5* were crossed with the wild type, the F_1 progeny showed no phototropism. The analysis of F_2 progeny indicated that the seedlings segregated for phototropic response or no phototropic response in 1:3 ratio respectively. These observations strongly suggest that *Nps-2* and *Nps-5* acts as dominant genes.

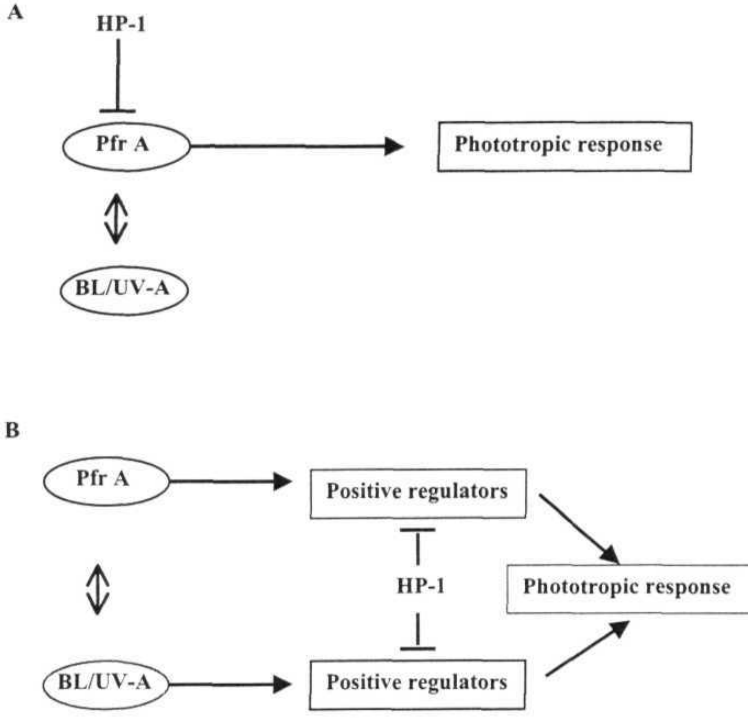

Figure.5.1. Possible mode of action of HP-1 in phototropic signal transduction pathway. A. HP-1 might act on phyA directly and thereby attenuates the transmission of light signal from phyA and its co-action with blue/UV-A photoreceptor or B. HP-1 down-regulates activity or expression of positive regulators of phyA-specific signal transduction or BL/UV-A specific signal transduction or common signal transducers of both phyA and BL/UV-A signal transduction pathways.

In general, the phenotype of dominant mutations is not as informative as the phenotype of recessive mutations particularly with respect to physiological meaning of the mutation. Mutants of dominant nature usually involve the functional inactivation of a wild-type gene product by coexpression of a mutant allele of that gene. Such an inactivation could result from inactivation of a multisubunit complexes through cross-oligomerization of normal wild type and defective mutant polypeptides. In literature several mechanisms have been invoked to explain dominant mutations: 1. Overproduction, or in rare cases underproduction, of a normal gene product. 2. Production of an inhibitory gene product (dominant negative) or 3. Production of a normal or an altered gene product at an incorrect time or place (Harberd and Freeling 1989; Scott, 1990). The fact that apical leaves of homozygous or heterozygous *Nps-2* shows epinasty (data not shown) strongly argues that overproduction of a normal gene product might be responsible for this effect. Further the present study suggests that these mutations are not dominant negative mutations since the presumptive wild type gene product does not affect auxin or ethylene sensitivity (Table 4.5).

Since gravitropism of hypocotyl and root are normal in these mutants, it is unlikely that the non-phototropic phenotype results from an alteration in the capacity of curvature itself (Table 4.5). If one assumes that the *Nps-2* and *Nps-5* mutants are impaired at a point in the phototropic signal transduction where the signal chain would act antagonistically on gravitropism then one would expect in the absence of phototropism, the gravitropism supercedes. On the other hand, if phototropic signal transduction is normal and overcomes gravitropism but phenotypically shows no gravitropic or phototropic curvature implicates that the mutations lie farther downstream from photoreceptor where the two responses share common elements. From the observations that the *Nps-2* and *Nps-5* mutants show only gravitropism (Table 4.5) when treated

simultaneously to phototropic and gravitropic stimuli, it is apparent that *Nps-2* and *Nps-5* mutations affect specifically phototropism and might lie prior to a point where the gravitropic and phototropic transduction pathways converge.

Previous genetic analysis of phototropic signal transduction pathway in *Arabidopsis* has resulted in isolation of non-phototropic mutants (*nph1* to *nph4*; Liscum and Briggs; 1995). These mutants are affected either in photoreceptor (*nph-1*) or signal transduction (*nph-2*, *nph-3)* or growth response *(nph-4).* The *Nps-2* and *Nps-5* mutants of tomato isolated in this work are good candidates for a mutation either in photoreceptor or downstream of photoreceptor in phototropic signal transduction, based on the finding that they retain normal gravitropic response and show normal photomorphogenic responses. Both in overall phenotype and hypocotyl elongation response to white light, the *Nps-2* and *Nps-5* mutants are different from the now well characterized photomorphogenic mutants of tomato (Kendrick et al., 1997). All of our results, including the sensitivity to growth hormones, are in accord with *Nps-2* and *Nps-5* being specifically involved in phototropic signal transduction pathway.

5.19 PHYSIOLOGICAL AND GENETIC COMPLEXITY OF PHOTOTROPIC SIGNAL TRANSDUCTION

The fact that phototropic curvature is a complex response should not be surprising given the diversity of light conditions plants are exposed to in the natural environment. First, the results of the present study demonstrate an increasing complex interaction between blue and red light mediated process in phototropism. Exposure to blue light initiates adaptive process of primary bending response and mediates secondarily de-sensitization like response. On the other hand, although red light cannot elicit the response, it induces

desensitization and enhancement that arises from the modulation by phytochrome. Moreover, the physiological observations described serve to firmly establish phytochrome as ancillary component in the signal transduction. Adding to this complexity **is the** fact that phototropic growth by white light depends not just on the separate effects through blue light photoreceptor and phytochrome but also on simultaneous interactions between these receptors.

Second, the complexity of phototropic system(s) reflected in complex fluence-response relationship and in complex and multiple red light effects on these relationships probably represents the ability of plants to express and optimize phototropism during early developmental stages. Zimmerman and Briggs (1963) hypothesized that first and second phototropic responses might be mediated by different pigment systems. The partial or complete disappearance of first and second positive responses in *au* and *fri* mutants does not support the Zimmerman and Briggs hypothesis. Furthermore, observation that in *Arabidopsis* only a single *NPH-1* gene encodes the putative phototropin photoreceptor (Huala et al., 1997) indicate that both first and second positive phototropism are basically mediated by single pigment system. However, the demonstration that cryptochromes can affect specifically first positive phototropism (Ahmad et al., 1998c) indicates that the response is complex and can be brought about by multiple pigment systems.

Third, the available genetic evidences indicate that a complex network leading from a number of sensory inputs to a differential growth response control the phototropism. Recent studies have shown that plants contains multiple photoreceptors and multiple pathways of light signal transduction (Bowler and Chua, 1994; Short **and Briggs, 1994;** Jenkins et al., 1995; Chamovitz and Deng, 1996; Furya and Schofer, 1996). The results of the

present study are consistent with either a single photoreceptor coupled to different transduction pathways or the existence of multiple photoreceptors with their own pathways. It is also clear that the multiple pathways initiated by these photoreceptors function independently of one another but at the same time are also capable of interacting additively and synergistically (Mohr, 1994; Casal and Boccalandro, 1995; Ahmad and Cashmore, 1996; Fuglevan et al., 1996; Poppe et al., 1996). The studies strongly suggest that activation of at least two photo-sensory systems, a blue light photo-sensory system and phyA-dependent system, precedes the development of phototropic curvatures suggesting that multiple signaling systems may regulate in the establishment of the phototropic response in tomato. Integration of physiological response outputs from these multiple sensory response systems provides a powerful means of rapidly and efficiently responding to alteration in environmental conditions. Therefore, the degree of co-action between these photoreceptors under natural conditions might depend on the magnitudes of the independent and the interdependent actions. While the integration of signals from blue light photoreceptor and phytochrome are recognized biochemically and molecular details of interaction remains to be elucidated. Moreover, it is equally evident that phytochrome B1 is dispensable indicating that other phytochromes might act redundantly to modulate phototropism. Unfortunately we have no mutants deficient in phyB2, phyC, phyD and phyE. Hence, it is difficult to make a general conclusion with respect to existence and overlap of signal transduction pathways for different phytochromes.

Finally, the inactivation *of HP-1* gene product may provide a basis for the ability of plants to respond to qualitative patterns of light signals. Further studies on the cloning and characterization *of HP-1* gene should elucidate how it elicits the light induced changes in phyA signaling pathway of phototropism.

It would also be of interest to determine whether only phytochromes or blue light photorcceptors are under the negative control by HP-1. We have also genetically identified two new mutants *Nps-2* and *Nps*-5, that are likely to be involved specifically in phototropic signal transduction. Further, complementation and epistasis analysis of these mutations will help to place them in the blue light mediated or phytochrome mediated signaling network. These mutants also provide a start toward the molecular-genetic analysis of the transduction steps leading from the initial photochemical reactions to the phototropic response.

SUMMARY

The dark-grown etiolated tomato seedlings were characterized for phototropic response to unilateral blue light. Phototropic dose response relationships, investigated revealed two response types: first and second positive curvatures resembling in fluence-dependence and kinetics of development previously described in other species. Prior exposure of seedlings with red light, which by itself was phototropically ineffective, enhanced the magnitude of first and second positive phototropic curvatures. It also reduced the time-threshold required to induce second positive response. This offers evidence for the hypothesis that at least two different photosystems are involved in phototropism, a blue/UV-A photoreceptor in inducing the response and red/far-red photoreceptors, phytochromes, in modulating the response.

Short-term irradiation from above with blue or white light alone or red or far-red light followed by blue light, immediately prior to unilateral phototropic stimulus eliminated subsequent first positive phototropism. However, blue light followed by red or far- red light from above induced first positive phototropism, whereas white light followed by red or far-red light could not induce the response. The data indicate that complex independent and interdependent interactions are involved between phytochromes and blue light photoreceptor in the sensitization and desensitization of first positive phototropism. Furthermore, compared to dark-grown seedlings, sensitivity to second positive phototropic stimulus decreases in de-etiolated seedlings depending on time and irradiation history of seedlings.

The roles of phytochrome A (phyA), phytochrome B1 (phyB1) in the control of blue light mediated phototropic response were investigated using photomorphogenic mutants of tomato that are affected in levels of different

phytochromes species: *au* (affected in chromophore biosynthesis possessing less than 5% levels of PHYA), *fri* (null for PHYA protein), *tri* (lacks PHYB1 apoprotein) and *trifri* (lacks PHYA,PHYB1 aproproteins). While to a single pulse of blue light both *au* and *fri* mutants showed highly reduced first positive curvatures, however, with multiple pulses of blue light treatment first positive curvatures increased in *au* compared to/n mutant. To continuous blue light, second positive phototropic response was impaired in *au* mutant and severely impaired in/n mutant and *trifri* double mutants; however, was normal in *tri* mutant. These evidences indicate that in tomato PHYA is essential for blue-light induced first and second positive phototropism and blue light photoreceptor is functionally dependent on it. On the contrary, there is no functional dependence of blue light photoreceptor on PHYB1, possibly due to redundancy of light stable pool of phytochrome species.

The photomorphogenic mutants were tested for their capacity to red-light-enhancement of phototropism, which is phytochrome-initiated response. In wild type, *hp-1* and *tri* seedlings, enhancement of first positive phototropism was perceived by R or FR or both; the *au* and *fri* single mutants and the *au,hp-1, fri,hp-1* and *tri,fri* double mutants showed insignificant enhancement compared to wild type. However, some increase in the level of first positive phototropic enhancement was observed in *au* and *fri* single mutants and *trifri* double mutants compared to their respective dark controls. It is concluded from these observations that along with PHYA other phytochromes also play a role in enhancement of first positive phototropism. Furthermore, since loss of PHYB1 had no significant effect, it has no significant role to play in enhancement of phototropism.

The *hp-1* mutant, shown to exaggerate for phytochrome responses and proposed to amplify a step in transduction chain, was used to dissect its involvement in the phototropism. In addition, the epistatic role *of HP-1* to act downstream of phytochromes was tested in its double mutants: *au,hp-1* and *fri,hp-1*. The *hp-1* mutant showed exaggerated phototropism for first and second positive curvatures. Moreover, the *hp-1* mutant showed extremely reduced time threshold of about 3-5 min to induce second positive response in dark-grown seedlings compared to wild type which needed 10 min and required a prior red or far red light pretreatment to reduce time threshold to 5 min. The *hp-1* mutation also restored second positive phototropism in the double mutants *au,hp-1* and *fri,hp-l* that is impaired in the *au* and *fri* mutants. These results suggest that *HP-1* wild type gene product acts downstream of the phytochrome photoreceptor and functions as a negative regulator in the transduction pathway of second positive phototropism.

Induction of mutations was done by chemical mutagenesis of seed and screening the harvested seed of M_2 generation. The dark-grown M_2 seedlings affected in phototropism were isolated by treating to continuous unilateral blue light and eight lines were found that are non-phototropic. Among them, two lines designated *Nps-2* (for *Non-phototropic seedling*) and *Nps-5*, showed dominant inheritance of the trait. In contrast to wild type, although the mutants showed no phototropism through out seedling development, light mediated hypocotyl elongation inhibition, hypocotyl and root gravitropism, and ethylene and auxin sensitivity were found to be normal. Furthermore, when treated simultaneously to light and gravity stimuli, the mutant seedlings showed gravitropic response compared to wild type which showed phototropic

response. This suggests that *Nps-2* and *Nps-5* mutants are specifically affected in the early steps of signal transduction pathway of phototropism.

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