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 $hp/l$ locus implied that this gene might encode product involved in the light regulatory steps (Peters et al., 1989). To better understand the function of $HP-l$ gene product epistatic study between $hp/l$ 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 $HP-l$ gene in the phototropism of tomato seedlings. Our observations that $hp/l$ mutant of tomato is consistent with the $HP-l$ 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 \( \mu \text{mol m}^{-2} \). In comparison, maximal first positive phototropic curvature of *Avena* found at approximately 0.1 \( \mu \text{mol m}^{-2} \) (Zimmerman and Briggs, 1963), that of alfalfa, pea, flax and mungbean at approximately 5.0 \( \mu \text{mol m}^{-2} \) (Baskin and Iino, 1987) and that of *Arabidopsis* at 0.5-1 \( \mu \text{mol m}^{-2} \) (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 correlation. 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 al., 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 (lino, 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 fluence 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 retention 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 \textit{au}, \textit{fri} and \textit{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 \textit{au} differs substantially from that of its wild type parent. The \textit{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 \textit{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 \textit{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 \textit{au} mutant is in general, consistent with its low (light labile) phytochrome levels. However, the chromosomal location of the \textit{au} mutation does not appear to correspond with the tomato phyA gene (Sharrock et al., 1988). One of the known effects of the \textit{au} mutation is the reduced type I phytochrome levels (Koornneef et al., 1985; Parks et al., 1987; Lopez-Juez et al., 1990). The \textit{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 \textit{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 \textit{au} mutant could be the result of its general phytochrome deficiency. Alternatively, the \textit{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 (Oelmueller 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 $\textit{fri}$. The insensitivity to unilateral blue light observed in $\textit{n}$ 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 $\textit{Arabidopsis}$ (Shinomura et al., 1996) and CAB gene expression induction (Hamazato et al., 1997).

The results obtained with $\textit{au}$ and $\textit{fri}$ 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 $\textit{Arabidopsis}$ (Janoudi et al., 1997). Apparently, it appears that phyA has a different function in tomato compared to $\textit{Arabidopsis}$. Characteristically, one feature distinguishes $\textit{au}$ and $\textit{fri}$ mutants from phy-deficient mutants of $\textit{Arabidopsis}$: 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 $\textit{Arabidopsis}$ both the phytochromes contribute to the phototropic response in blue light. However, after 2 hr seedlings do show curvature, whereas in $\textit{n}$ mutant of tomato seedlings second positive is lost completely. It is possible that the existence of redundancy between phyA and phyB in $\textit{Arabidopsis}$ might have been responsible for the differences observed.
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 an influence of phytochrome on overcoming the 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 (Steinitz 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 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).

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 *Fagopyrum*. 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 PhyA 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 reductase, 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-l* 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-l* 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-l* gene product, which might impede the phototropic signal pathway or alternatively *HP-l* might convey hypersensitivity to phytochrome.

The *fri,hp-l* double mutant shows second positive phototropic response similar to wild type, demonstrating that the *fri* mutation is not completely epistatic to *hp-l*. Similarly *au,hp-l* 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-l* mutant is also seen in the *au,hp-l* double mutant. The interaction of the *au* and *fri* with *hp-l* in double mutants (Figs 4.11 and 4.13) suggests the operation of phytochrome pathway controlling seedling phototropism and the *hp-l* product is to be down-regulated for phyA-mediated phototropic response. In a formal genetic sense, *HP-l* 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-l* restores second positive phototropism in *au,hp-l* and *fri,hp-l* 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-l* and *fri,hp-l* regains second positive phototropism, the possibility exists that phytochromes other than light labile pool might enhance the response in the absence of *HP-l* 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-l* 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-l* 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-l* gene product and both B and the *hp-l* mutation appear to be able to relieve this constraint (Peters et al., 1989, 1992b).
On the other hand, phenotype of \textit{hp-1} appears to be identical to those obtained by ecotopic expression of PHYA in tomato (Boylan and Quail, 1989) and \textit{in vivo} spectrophotometric and \textit{immunochemical} analysis failed to provide evidence that the \textit{hp-1} mutant is a photoreceptor mutant (Peters et al., 1992b; Kerckhoffs et al., 1997a). Conversely, double mutant analysis of \textit{hp-1} with Phy A and PhyB-1 deficient tomato mutants has demonstrated that the \textit{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 \textit{hp-1} mutation affect fairly specifically the responses mediated by phytochrome.

It is proposed that the \textit{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 \textit{hp-1} is recessive and mimics its action in double mutants due to response amplification, it is imperative to suggest that wild type \textit{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, \textit{hp-2}, is known to be similar to \textit{DET-2} negative regulator of photomorphogenesis in \textit{Arabidopsis} (Mustilli et al., 1999) further supporting the negative regulation by \textit{HP-1} in phototropic response of tomato.

5.17 RELATIONSHIP OF PHY A, BL/UV-A PHOTORECEPTOR AND \textbf{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
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 (*nph*1 to *nph*4; 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 contain 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 photoreceptors are under the negative control by HP-1. We have also genetically identified two new mutants \textit{Nps-2} and \textit{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.