PHOTOMORPHOGENETIC MUTANTS OF HIGHER PLANTS

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PHOTOMORPHOGENETIC MUTANTS OF HIGHER PLANTS

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op vrijdag 19 juni 1992
des namiddags te half twee in de Aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

(1). Ondanks het feit dat phy A en phy B dezelfde fotomorfogenetische processen kunnen reguleren, zijn ze niet volledig onderling uitwisselbaar.

Boylan en Quail (1989) The Plant Cell 1, 765-773; Wagner et al. (1991) The Plant Cell 3, 1275-1288; dit proefschrift

(2). De *hp* mutatie in tomaat heeft invloed op een amplificatiestap in de fytochroom transductieketen.

dit proefschrift

(3). Hoewel de kans bestaat dat de interpretatie van de resultaten die voortvloeien uit onderzoek met fotomorfogenetische mutanten moet worden aangepast vanwege de ontdekking van het bestaan van steeds meer fytochroomtypen, is het nuttig dergelijk onderzoek te blijven doen naast het in kaart brengen van de verschillende fytochroomtypen die in de betreffende planten aanwezig zijn.

Pratt et al. (1991) In: Phytochrome Properties and Biological Action (Eds. B. Thomas and C.B. Johnson) pp. 39-55

(4). De regulatie van genexpressie kan zich op post-transcriptioneel niveau afspelen via de stabiliteit van het mRNA. Onderzoek naar de rol van eiwit-RNA interacties bij de regulatie van mRNA stabiliteit in het cytoplasma is daarom waardevol.

(5). Het is aanbevelenswaardig artikelen betreffende samenlevingsvormen tussen dieren en chloroplasten vaker in botanische tijdschriften op te nemen.

Ben-Izhak Monselise en Rabat (1980) Israel Journal of Zoology 29, 125-128

(6). Dat amalgaamvullingsmateriaal wel in iemands gebit doch niet in het oppervlaktewater mag worden gestort, is merkwaardig.

(7). Het boek waarin de complete sequentie van het genoom van de nematode *Caenorhabditis elegans* wordt beschreven, zal tot de saaiste van de in de toekomst te verschijnen boeken behoren.

(8). Als wetenschap is kunstgeschiedenis zoiets als het vangen van vlinders met een lineaal.

Uitspraak van G.IJ. de Wit, opgetekend door W. Knaap

(9). Wanneer de overheid serieus het autogebruik wenst te beteugelen, zou zij maatregelen kunnen overwegen zoals ze die getroffen heeft om het roken terug te dringen: alle automobielen voorzien van een waarschuwende tekst ("Autorijden schaadt uw en mijn gezondheid") en het autorijden in de openbare ruimte verbieden.

(10). Afschaffing van de OV-jaarkaart voor studenten en dienstplichtigen zou het rechtvaardigheidsgevoel van de kaartjeskopende treinreiziger zeer ten goede komen, daar deze dan niet meer hoeft te constateren dat hij niet alleen zijn eigen vervoersbewijs, maar middels zijn belastingafdracht ook dat van veel andere reizigers in de coupé voor zijn rekening neemt.

(11). Als de bewoners van Schiermonnikoog verdere ondergraving van hun eiland willen voorkomen, zouden zij er goed aan doen vaker konijn op de menukaart te zetten.

(12). Wetenschappers zouden meer acht moeten slaan op het verschijnsel Karoshi.

Stellingen behorende bij het proefschrift 'Photomorphogenetic mutants of higher plants' door Janny L. Peters, in het openbaar te verdedigen op vrijdag 19 juni 1992, te Wageningen.

'Alsof geld wichtiger is, dan duchtiger wetenschappelijker arbeid!'
Professor Zbigniew Prlwytzkofski.

Ter herinnering aan mijn opa,
Gerhard Johan Peters.

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PHYTOCHROME CONTENT AND HYPOCOTYL GROWTH OF LONG HYPOCOTYL MUTANT AND WILD-TYPE CUCUMBER SEEDLINGS DURING DE-ETIOLATION

GENERAL DISCUSSION

ABSTRACT

Thesis of Janny L. Peters (1992): Photomorphogenetic mutants of higher plants. Department of Plant Physiological Research, Wageningen Agricultural University, Generaal Foulkesweg 72, NL-6703 BW Wageningen, The Netherlands.

In this thesis the hypothesis that different molecular types of phytochrome have different physiological roles has been elaborated and an attempt has been made to assign functions to these phytochrome types. For this purpose a genetic approach was chosen and the following potential phytochrome mutants were used: the tomato *aurea (au)* mutant; the cucumber long-hypocotyl *(lh)* mutant and the *Arabidopsis* long-hypocotyl *(hy3)* mutant. Comparing the response of the mutant affected in the abundance or function of a particular phytochrome type with that of its isogenic wild type shows directly the relevance of the phytochrome type concerned. The *au* mutant is proposed to lack the light-labile phytochrome type (phy A), while normal levels of the light-stable phytochrome type (phy B) are present. This results in a reduction of: (i) the inhibition of the hypocotyl growth; (ii) the anthocyanin synthesis; (iii) the chlorophyll content, but a normal end-of-day far-red (EOD FR) response. In the *lh* and *hy3* mutants the opposite situation appears to be found: normal levels of phy A, but a lack of phy B. Both the *lh* and *hy3* mutant do not show the EOD FR response, while the hypocotyl growth in continuous R and B is less inhibited when compared to the wild type. Therefore it is proposed that phy B plays a role in the EOD FR response, while both phy A and phy B are important for the inhibition of the hypocotyl growth. With the help of other mutants described in the literature a more detailed assignment of functions to the different phytochrome types is proposed.

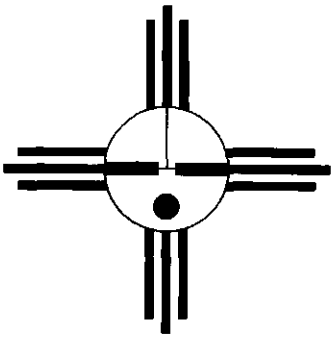
In addition, a potential phytochrome signal transduction mutant, the tomato high-pigment *(hp)* mutant, was characterized. This mutant exhibits exaggerated phytochrome responses, *e.g.* high anthocyanin synthesis and short hypocotyl length when compared to wild type. It is proposed that the *hp* mutation affects an amplification step in the phytochrome transduction chain.

Key words: anthocyanin; *Arabidopsis thaliana*; cucumber; *Cucumis sativus*; end-of-day far-red light response; phytochrome; hypocotyl growth; *Lycopersicon esculentum*; mutants; photomorphogenesis; phytochrome; tomato.

LIST OF ABBREVIATIONS

A535	absorbance at 535 nm
au	*aurea* mutant of tomato
B	blue light
cab	gene coding for clorophyll *a/b* binding protein
cop	constitutive-photomorphogenic mutant of *Arabidopsis*
cv	cultivar
D	dark(ness)
det	de-etiolated mutant of *Arabidopsis*
DET	product of *Det* gene
dg	dark-green mutant of tomato
EOD	end-of-day
EMS	ethylmethanesulphonate
FR	far-red light
fr wt	fresh weight
GA	gibberellic acid
H	phytochrome cycling rate
HIR	high irradiance response
HP	product of *Hp* gene
I	intermediate
lh	long-hypocotyl mutant of cucumber
lv	long-stemmed mutant of pea
hp	high-pigment mutant of tomato
hy	long-hypocotyl mutant of *Arabidopsis*
$hy3^{TAG}$	met T-DNA getagde *hy3* mutant of *Arabidopsis*
LFR	low fluence response
λ_{max}	peak transmission wavelength
ma_3^R	maturity mutant of sorghum
nos	gene coding for nopaline synthetase
nptII	gene coding for neomycin phosphotransferase II
PI	light-labile phytochrome type
PII	light-stable phytochrome type
PAL	phenylalanine ammonia lyase
pet E	gene coding for plastocyanin
phy	gene coding for phytochrome
PHY	immunochemically detectable product of *phy* gene
phy	PHY with active chromophore
Pfr	FR-absorbing form of phytochrome
Pr	R-absorbing form of phytochrome
psa D	gene coding for subunit II of photosystem I
Ptot	total phytochrome (Pr+Pfr)
φ	phytochrome photoequilibrium (Pfr/Pr+Pfr)
pro	*procera* mutant of tomato

R	red light
rbcS	gene coding for the small subunit of rubisco
rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SE	standard error
T-DNA	transfer deoxynucleic acid
UV	ultraviolet light
VLFR	very low fluence response
WS	*Arabidopsis* Wasselewskija
WT	wild type
WL	white light
yg	yellow-green mutant of tomato



CHAPTER 1

INTRODUCTION

Phenomena whereby plants sense and respond to the light environment are collectively called photomorphogenesis, literally, the development *(genesis)* of form *(morpho)* of an organism as influenced by light *(photo)*. The effect of light on morphogenesis is well illustrated by comparison of a dark (D)-grown (etiolated) seedling with a light-grown (de-etiolated) seedling (Fig. 1.1). Since both seedlings have been reared from genetically identical seeds it can be concluded that light regulates processes such as expansion of cotyledons and leaves, inhibition of hypocotyl growth and stimulation of pigment synthesis.

The primary cause of this rather dramatic change, is the capture of light by photoreceptor(s) and the relay of information *via* a chain of events (transduction chain), which eventually leads to the responses. At least three different photomorphogenetic photoreceptors have been distinguished in plants: phytochrome, a blue light (B)/UV-A photoreceptor, sometimes called cryptochrome, and a UV-B photoreceptor (Mohr, 1986). Only phytochrome has been isolated and has consequently been the most extensively studied.

1.1. PHYTOCHROME

Based on the red light (R)/far-red light (FR) reversible induction of seed germination the existence of a pigment was postulated, which is inter-convertible by R and FR (Borthwick *et al.*, 1952). Subsequently this blue pigment was detected spectrophotometrically and named phytochrome (Borthwick and Hendricks, 1960).

1.1.1. Photochemical and non-photochemical reactions

Phytochrome exists as two photoisomers, the R-absorbing (Pr) and the FR-absorbing (Pfr) form. Irradiation with R converts Pr to Pfr, which can be converted back to Pr by FR irradiation:

$$Pr \underset{FR\ (730\ nm)}{\overset{R\ (660\ nm)}{\rightleftharpoons}} Pfr$$

This photoconversion occurs *via* several intermediates (Schaffner *et al.*, 1991). Since the absorption spectra of Pr and Pfr overlap considerably in the R region (Fig. 1.2) no pure Pfr can be obtained and saturating irradiation leads to continuous interconversion between Pr and Pfr resulting in a dynamic photoequilibrium (φ = Pfr/Ptot, where Ptot is total phytochrome = Pr+Pfr). The relative proportion of the two photoisomers varies depending on the spectral distribution of incident light. In this thesis the photoequilibrium is calculated using the formulae of Mancinelli (1986) and the molecular extinction coefficients and quantum yield data (number of molecules affected as proportion of the number of quanta absorbed) from Lagarias *et al.* (1987). This leads to a maximum for φ of about 88% in R. Further enrichment of Pfr can be obtained by immunopurification (Cordonnier, 1989). Estimation of the true state of phytochrome *in vivo* is complicated, because in addition to the photochemical reactions mentioned above, non-photochemical D reactions play a role in determining the photoequilibrium. These D reactions include: (i) *de novo* synthesis of Pr, which itself is inhibited by Pfr; (ii) destruction of Pfr, probably *via* the ubiquitin-dependent proteolytic pathway (Hatfield and Vierstra, 1990) and (iii) D reversion of Pfr to Pr (Fig. 1.3).

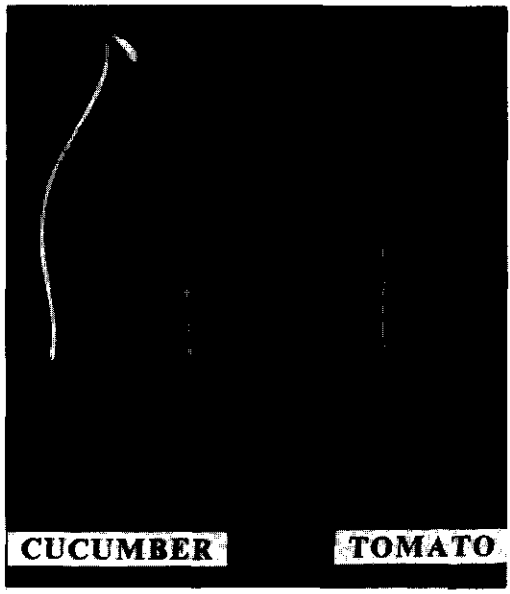


Figure 1.1. Cucumber and tomato seedlings grown for 5 d in 16 h white light / 8 h dark cycles (right) and continuous darkness (left).

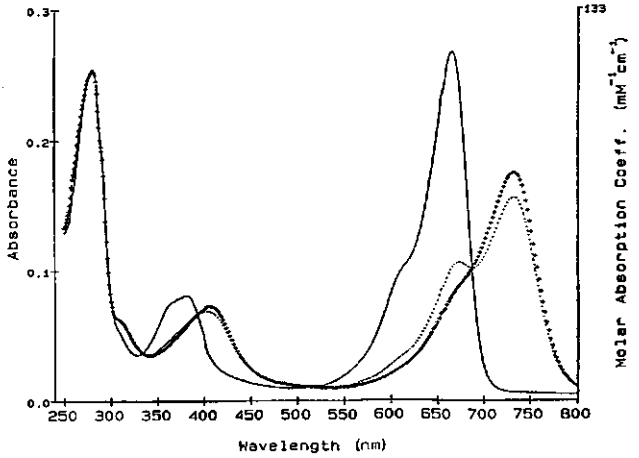


Figure 1.2. Absorption spectrum of 124-kDa rye phytochrome at 5°C. The spectra of the Pr form (*i.e.* FR photoequilibrium mixture which is about 2% Pfr and 98% Pr) (——), the Pfr form (*i.e.* R photoequilibrium mixture which is about 88% Pfr and 12% Pr) (· · ·) and that calculated for 100% Pfr (+++) are shown. Adapted from Lagarias *et al.* (1987).

1.1.2. Phytochrome response modes

The proposal that Pfr is the biologically active form of phytochrome was based on the extreme sensitivity of D-grown plants to fluences of R, which establish only a minor conversion of Pr to Pfr (Hendricks, 1964). Since this means a relatively large increase in Pfr (from zero to a detectable value) and a relatively small decrease in Pr, the plant is likely to respond to the appearance of Pfr and not to the disappearance of Pr. In some cases a positive correlation between Pfr concentration, [Pfr], and response has been found (*e.g.* Mandoli and Briggs,

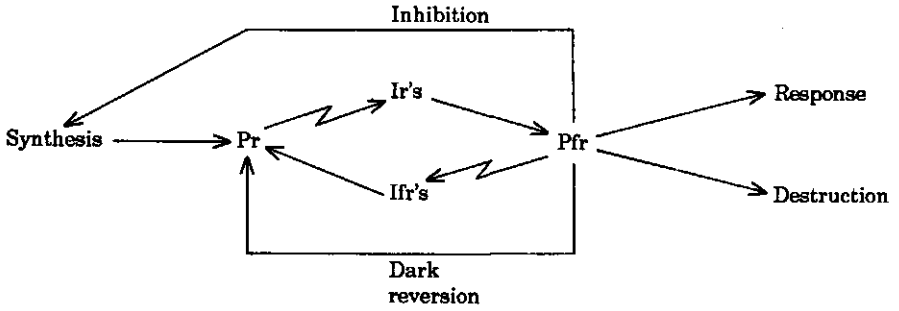


Figure 1.3. The phytochrome system. The synthesis of phytochrome, as Pr, is inhibited by Pfr, while the photoconversion between Pr and Pfr occurs *via* several intermediates (Ir's and Ifr's). Eventually Pfr leads to biological activity and can disappear *via* D reversion to Pr and proteolytic destruction of its protein moiety.

1981; Drumm and Mohr, 1974), while in other cases there seems to be a better correlation with Pfr/Ptot (Smith, 1981; 1983; Child and Smith, 1987). If the phytochrome concentration remains stable, [Pfr] and Pfr/Ptot are proportional. However, if the phytochrome regulating the response is light labile as Pfr, [Pfr] and Ptot could be different. Since the photoequilibrium, Pfr/Ptot, is independent of the phytochrome concentration it is a reliable means to detect the spectral composition of the light. From an ecological point of view, the R:FR photon ratio is an important parameter, since shading by vegetation (Casal and Smith, 1989) and the presence of neighbouring plants (Ballaré *et al.*, 1990) affect this ratio, which *via* the changed state of phytochrome leads to an increase in stem extension. The magnitude of this increase depends upon the species concerned, *i.e.* woodland or shade-tolerant species are less responsive than non-woodland or shade-avoiding species (Morgan and Smith, 1979). Irradiation, *via* [Pfr] and/or Pfr/Ptot, leads to several very divergent responses, which are often classified according to the quantity of light required: very low fluence responses (VLFR), low fluence response (LFR) and high irradiance responses (HIR).

1.1.2.1. Very low fluence responses. Since saturation of these responses is reached at extremely low R fluences (10^{-4}-10^{-1} μmol m^{-2}) and Pfr concentrations (10^{-6}-10^{-3} Pfr/Ptot) (Kendrick and Kronenberg, 1986), FR establishes sufficient Pfr to induce such responses. This means that the responses are not R/FR reversible. However, action spectra suggest that phytochrome is the photoreceptor in VLFRs. Examples of VLFRs are: induction of germination of some seeds (Cone *et al.*, 1985) and inhibition of seedling extension growth (Blaauw *et al.*, 1968; Mandoli and Briggs, 1981). In practice, many VLFRs are often overlooked, because of the use of green 'safelight' during plant handling in 'D'.

1.1.2.2. Low fluence responses. This group includes most of the classical R/FR reversible responses, they require about 1-1000 μmol m^{-2} R and are fluence dependent until photoequilibrium is reached. Once the fluence is high enough to reach photoequilibrium the LFR is saturated. The Bunsen-Roscoe law of reciprocity, *i.e.* a response is independent of irradiation time or fluence rate (μmol m^{-2} s^{-1}) at a given total fluence (μmol m^{-2}), holds for LFRs.

1.1.2.3. High irradiance responses. A number of responses have a requirement for continuous irradiation. After the establishment of photoequilibrium these HIRs, in contrast to LFRs, remain fluence-rate dependent. Attempts to explain the fluence-rate dependency of the HIR generally emphasize the importance of the rate of photochemical turnover or cycling (H) between Pr and Pfr, which is a function of fluence rate. The Bunsen-Roscoe law of reciprocity does not hold for HIRs. The action spectra of HIRs show considerable variation, depending on *e.g.* plant species and plant age, but maxima are mostly present in the FR, B and UV regions with a shift towards the R region in light-grown plants (Beggs *et al.*, 1980). Although HIRs are not R/FR reversible, bichromic experiments have shown that at least FR-HIRs are mediated by phytochrome (Hartmann,

1966). The FR maximum has been explained on the basis of the Pfr/Ptot ratio which results in the maintenance of the highest amount of Pfr integrated over the time period of irradiation.

Smith *et al.* (1991) further distinguish response modes in fully de-etiolated plants, which may be different from those shown by etiolated plants: photoperiod perception, end-of-day responses (Downs *et al.*, 1957) and R:FR photon ratio perception.

1.1.3. Types of phytochrome

The complexity of photomorphogenesis, with its different response modes and its diversity of responses, has been extremely difficult to reconcile with the initial assumption that only a single molecular species of phytochrome exists. However, physiological, spectrophotometrical and immunochemical evidence has now accumulated which suggests that multiple types of phytochrome exist (for review see Furuya, 1989).

As mentioned above, Pfr is the biologically active form of phytochrome which is unstable in D due to destruction and D reversion to Pr. Therefore a correlation would be expected between the physiologically R/FR reversible responses and the status of phytochrome detected in the plant. However, in some R/FR reversible responses separation between the R and FR irradiation by a D period sufficiently long to lead to Pfr disappearance still results in reversibility of the response. Such an extended FR reversibility can only occur when the Pfr mediating the response is stable during the D period. Spectrophotometrical measurements of Pfr destruction showed that the Pfr detected in light-grown plants is much more stable than the bulk of phytochrome in etiolated seedlings and that the Pfr disappearance after R irradiation in etiolated seedlings occurs with biphasic kinetics (Jabben and Holmes, 1983). This indicated that besides the bulk Pfr pool characterized by fast destruction (half life of approximately 1 h at physiological temperature), a pool of phytochrome characterized by slower Pfr destruction kinetics exists in both light- and D-grown plants (half life between 5 h and complete stability). Therefore it was proposed that two physiological and spectrophotometrical distinct phytochrome pools exist: one which is synthesized in the Pr form in D and decreases rapidly in light as Pfr (the light-labile type or PI) and another which is relatively stable as Pfr and is present in both D-grown seedlings and light-grown plants (the light-stable type or PII). The light-labile type is abundant in etiolated tissue, while the light-stable type dominates in green light-grown plant tissue. The existence of these different phytochrome types has been confirmed with the aid of antibodies (Tokuhisa *et al.*, 1985; Shimazaki and Pratt, 1985 and Abe *et al*, 1985). Recently it was shown by Wang *et al.* (1991) that green oat leaves contain at least three phytochromes, only one of which is abundant in etiolated tissue.

Physicochemical comparison of the different phytochrome types revealed differences in: absorbance and difference spectra, monomer molecular weights and peptide maps upon digestion with endoproteases. This suggests that they are either products of different genes or the result of post-translational

modification. The first evidence that the different phytochrome pools are products of different phytochrome genes *(phy)* came from work with pea (Abe *et al.*, 1989), followed by work with *Arabidopsis* (Sharrock and Quail, 1989) and oat (Pratt *et al.*, 1991). In this thesis the following terminology is used: PI for the physiologically predicted and spectrophotometrically detected light-labile phytochrome type, PHYA for the immunochemically detected protein encoded by the *phyA* gene and phy A for the photochemicallly active phytochrome after incorporation of functional chromophore. In *Arabidopsis* four to five phytochrome-related gene sequences were recognized, three of which *(phyA, B* and *C)* were actually isolated and sequenced (Sharrock and Quail, 1989). Deduction of the amino-acid sequence revealed that one of the proteins (PHYA) had strong similarity to etiolated-tissue phytochrome proteins described in other plant species, while the other two (PHYB and PHYC) were unique when compared to each other, PHYA and to previously described phytochromes. In *Arabidopsis*, as well as rice, *phyA* is negatively regulated by phytochrome, while *phyB* is constitutively expressed irrespective of light treatment (Sharrock and Quail, 1989; Dehesh *et al.*, 1991). This is reminiscent of the behaviour of the labile (PI) and stable (PII) phytochrome types and it is tempting therefore to speculate that *phyA* encodes PI and *phyB* a component of the PII pool.

Since phytochrome is now known to be represented by a family of genes encoding different molecular types of the photoreceptor, it is conceivable that these photoreceptors have different physiological roles. In this thesis this concept is elaborated and an attempt made to determine the physiological roles of the different members of the phytochrome family. For this purpose phytochrome mutants are useful, because comparison of the response of a mutant, affected in the abundance or function of a particular phytochrome type, with that of its isogenic wild type should show directly the relevance of the phytochrome type concerned.

1.1.4. Properties of the phytochrome molecule

Isolation and purification of the intact phytochrome molecule from etiolated plant material (Vierstra and Quail, 1983) made study of its structure possible. The phytochrome monomer consists of a linear tetrapyrrole chromophore covalently linked to the apoprotein *via* a cysteine residue in the N-terminal domain (Lagarias and Rapoport, 1980). In solution, native phytochrome dimerizes (Lagarias and Mercurio, 1985) and the quarternary structure of this dimer appears to resemble a Y-shaped assembly, as indicated by electron-microscope (Jones and Erickson, 1989) and small-angle X-ray scattering studies (Tokutomi *et al.*, 1989; Nakasako *et al.*, 1990). These studies resulted in a two-domain model, in which there is a separation of the non-chromophore and chromophore domains. Recently, a more detailed structural domain model based on homology analysis of six phytochrome sequences has been proposed by Parker *et al.* (1991) (Fig. 1.4).

The chromophore is known to undergo a Z→E double-bond isomerization upon R irradiation (Rüdiger, 1986). However, this change in stereometric configuration of the chromophore is not sufficient to cause the shift to 730 nm

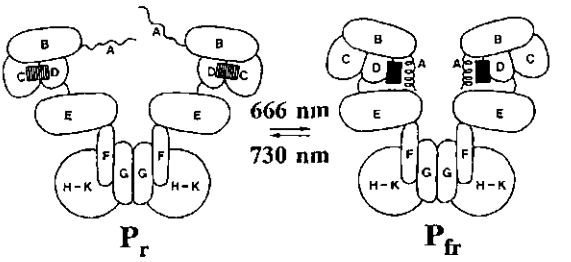


Figure 1.4. Structural model of phytochrome based on homology analysis of six phytochrome sequences. The chromophore domain contains regions A-E and the non-chromophore domain contains regions G-K, region F being the linker region. The solid rectangle represents the chromophore. A possible movement of the domains after R irradiation is illustrated. Adapted from Parker *et al.* (1991).

and some form of chromophore-protein interaction is presumably involved (Partis and Thomas, 1991). Comparative studies of Pr and Pfr tested the accessibility of the chromophore and revealed that the chromophore becomes more exposed in the Pfr form. Moreover, the 6-kDa N-terminal segment of phytochrome undergoes a more drastic change in conformation and orientation than any other part of the polypeptide (see for review Partis and Thomas, 1991). The photoreversible increase in α-helical folding of the apoprotein observed upon Pr to Pfr phototransformation probably occurs in the 6-kDa N-terminal portion of the protein (Vierstra *et al.*, 1987). Loss of the 6-kDa N-terminal portion of the protein results in a shift of λ_{max} for Pfr from 730 to 722 nm (Vierstra and Quail, 1982) and the N-terminal sequence is known to interact with the phytochrome chromophore in the Pfr form (Vierstra and Quail, 1983; Chai *et al.* 1987). Therefore it is possible that this interaction with the chromophore induces an increase in α helicity in the N-terminus chain (Parker *et al.*, 1991) and that it is the chromophore-protein interaction involved in the shift to 730 nm upon R irradiation.

The expression systems recently developed for production of the phytochrome protein (Lagarias *et al.*, 1991; Furuya *et al.*, 1991) may help in further clarifying the nature of the chromophore-protein interaction during phototransformation. Moreover, with such a system it will be possible to produce and study other phytochrome types besides that from etiolated plants studied until now.

1.1.5. Phytochrome transduction chain

As mentioned above, extensive research has concentrated on identifying differences between Pr and Pfr, because Pfr acts as the first element in the chain of cellular events leading to physiological responses. Although it is clear that the phytochrome molecule, in particular the N-terminal domain, undergoes conformational changes upon irradiation, there is no evidence as to which part of the molecule is involved in generating the initial signal(s) and how these

signal(s) are transduced within the cell to bring about the final responses. An attractive hypothesis is that phytochrome is an enzyme (*e.g.* protein kinase), with Pfr being the active configuration. However, the detected kinase activity is not an intrinsic property of phytochrome, but had to be ascribed to a co-purified kinase enzyme (Kim *et al.*, 1989; Grimm *et al.*, 1989). Research on the signal transduction chain has focussed mainly on phytochrome-mediated modification of membrane properties and gene expression.

Phytochrome is unlikely to be an intrinsic membrane protein and the bulk of phytochrome in the cell exhibits the properties of a soluble protein. However, there are several localized hydrophobic regions which could allow phytochrome to interact with membranes or membrane proteins (Partis and Grimm, 1990) and phytochrome indeed has the ability to associate with membranes (Roux, 1986; Terry *et al.*, 1989; Eisinger *et al.*, 1989). Recent research on the phytochrome-regulated swelling of isolated protoplasts revealed that this response was dependent on external Ca^{2+} and that there is evidence for the involvement of G-proteins, protein kinases and inositol trisphosphate turnover (Bossen, 1990). Perhaps Pfr leads, *via* the phosphatidyl-inositol pathway, to the opening of Ca^{2+} channels in the plasma membrane, resulting in enhanced transport of Ca^{2+} into the cytoplasm, where Ca^{2+} and calmodulin could act as second messengers in the phytochrome transduction pathway. Together with other reports (Chandok *et al.*, 1991; Hartmann and Pfaffman, 1990; Dürr and Scheuerlein, 1990) this suggests that signal transduction mechanisms comparable to those in animal and other plant regulatory systems may also be involved in the phytochrome transduction chain.

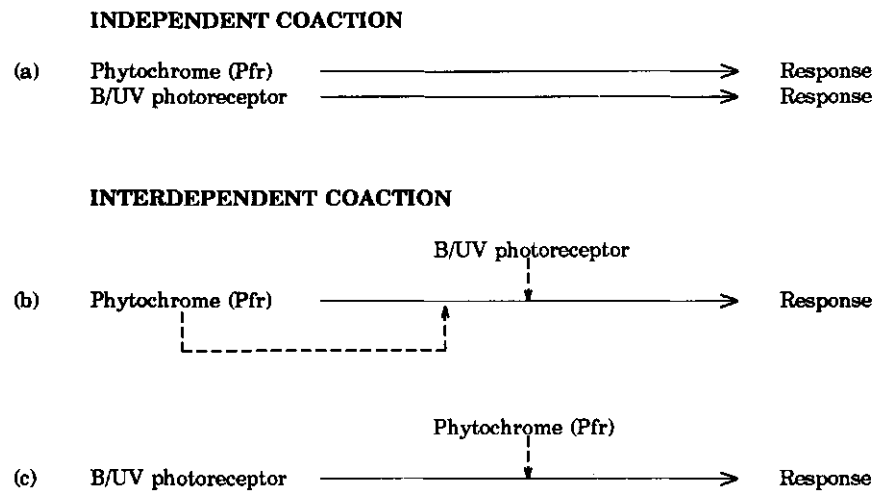


Figure 1.5. Possible modes of coaction between phytochrome and B/UV photoreceptor. The dashed lines indicate that the effectiveness of Pfr (b) or B/UV photoreceptor (c) in mediating the response can be increased by light absorption by the B/UV photoreceptor or phytochrome, respectively. See text for further explanation. After Mohr (1987).

The expression of several genes, *e.g.* genes coding for phytochrome *(phy)*, chlorophyll *a/b*-binding protein *(cab)*, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase *(rbsS)*, have been reported to be up or down regulated by phytochrome at the transcriptional or post-transcriptional level (Jenkins, 1988; Thompson, 1991). The transcriptional control of light-regulated gene expression involves the binding of activators or repressors to regulatory elements of the gene. Several of these regulatory or *cis*-acting DNA sequence elements in the upstream region of the genes and some of the protein factors, also called *trans*-acting elements, which bind to these sequences have been identified (Gilmartin *et al.*, 1990; Schindler and Cashmore, 1990; Jenkins, 1991). Whether the phytochrome-regulated gene expression and modification of membrane properties act independently is unclear.

A new approach in elucidating parts of the phytochrome transduction pathway is the isolation of genetic mutants. In this thesis a possible phytochrome transduction-chain mutant, the high pigment *(hp)* mutant of tomato, will be characterized and extensively discussed.

1.2. BLUE LIGHT AND UV PHOTORECEPTORS

Although B and UV can be absorbed by phytochrome, there is ample evidence for the existence of specific B/UV-A and UV-B photoreceptor(s) (Mohr, 1986). To date, no chemically defined B/UV photoreceptors have been identified, but flavoproteins are considered the most likely candidates (Galland and Senger, 1988a), although carotenoproteins (Song, 1987) and pterins (Galland and Senger, 1988b) are also candidates. Blue light plays a role in directional responses (*e.g.* phototropic curvature), morphogenetic responses (*e.g.* inhibition of the hypocotyl length) and metabolic responses such as biosynthesis of pigments (*e.g.* anthocyanin) (for review see Senger, 1987). Since phytochrome can also absorb B and UV, it has been difficult to exclude the possibility that phytochrome is involved in many B-mediated responses.

1.2.1. Interaction between the different photoreceptors

Both phytochrome and B/UV photoreceptor(s) play a role in the control of the responses studied in this thesis: hypocotyl growth and anthocyanin synthesis (Attridge *et al.*, 1984; Cosgrove, 1986; Mancinelli, 1989; Mancinelli *et al.*, 1991). The mode of coaction between these photoreceptors is unknown and can be divided into two classes: independent and interdependent coaction (Mohr, 1987). The first possibility implies that the different photoreceptors operate independently of each other, eventually leading to the same response (Fig. 1.5a). For interdependent coaction the photoreceptors depend on some form of interaction to bring about the final response (Fig. 1.5b and c). Data supporting two types of interaction have been proposed: the 'responsiveness theory' (Fig. 1.5b) mainly presented by Mohr and coworkers (Mohr, 1986; Elmlinger and Mohr, 1991) implies that the only effect of B/UV is to establish or enhance the

responsiveness towards Pfr, while the 'presence theory' (Fig. 1.5c) implies that the B/UV photoreceptor requires only the presence of a certain amount of Pfr for its action to be expressed (Gaba and Black, 1987). The fact that irradiation of plants with B/UV always leads to photoconversion of some Pr to Pfr complicates studies on photoreceptor involvement and interaction in mediating responses to B and UV (Gaba and Black, 1987). The 'light equivalence principle method', *i.e.* light inducing similar φ and H in plant tissues will have similar biological effects if phytochrome is the only photoreceptor playing a role (Schäfer and Haupt, 1983) and the 'dichromatic irradiation method', *i.e.* comparison of the effects of light treatments differing in the state of only one of the two photoreceptors (Mancinelli *et al.*, 1991), are methods to determine the involvement and interaction of photoreceptors. The availibity of mutants altered in specific B responses (Khurana and Poff, 1989; Liscum and Hangarter, 1991) together with phytochrome mutants (Kendrick and Nagatani, 1991) allows a new approach to separate the functions of phytochrome and the B photoreceptor. Moreover, B/UV photoreceptor mutants could be helpful in the identification and isolation of the B/UV photoreceptors.

1.3. CHARACTERIZATION OF THE MUTANTS USED IN THIS STUDY

As mentioned above, mutants are playing an increasingly important role in unravelling the complex process of photomorphogenesis. The mutants used in this study were provided by Dr. M. Koornneef and coworkers (Department of Genetics, Wageningen Agricultural University, The Netherlands).

1.3.1. Tomato *aurea* mutant

During selection of gibberellin (GA)-responsive mutants, a mutant was isolated that required GA for germination, but in contrast to GA-deficient mutants was characterized by a long hypocotyl and a marked reduction in chlorophyll content when grown in white light (Koornneef *et al.*, 1981). A genetic analysis revealed that this recessive mutant was allelic with a previously described *aurea (au)* mutant (Koornneef *et al.*, 1985). This gene is located on chromosome 1 (Khush and Rick, 1968). Another mutant at the *au* locus has been isolated in the progeny of tomato plants derived from tissue culture by Lippuci di Paola *et al.* (1988).

The *au* mutant is one of the best characterized photomorphogenetic mutants of higher plants. It does not contain spectrophotometrically (Koornneef *et al.*, 1981, 1985) and immunologically (Parks *et al.*, 1987; Oelmüller *et al.*, 1989) detectable phytochrome in etiolated tissues. However, light-grown tissues of the *au* mutant contain about 60% of the *in vivo* (Adamse *et al.*, 1988b) and *in vitro* (López-Juez *et al.*, 1990b) spectrophotometrically detectable phytochrome compared to wild type, suggesting that the light-stable phytochrome type is present (PII).

Parks *et al.* (1987) and Sharrock *et al.* (1988) investigated the nature of the *au* lesion and showed that similar amounts of phytochrome mRNA are produced in the *au* mutant and the wild type and that this mRNA is functional in an *in vitro* translation system, yet *in vivo* the protein fails to accumulate. Therefore the deficiency of spectrophotometrically active phytochrome in the *au* mutant appears not to result from a lack of phytochrome gene expression. In addition, there appear to be multiple genes coding for phytochrome in tomato (Sharrock *et al.*, 1988; Hauser and Pratt, 1990, 1991). One of the tomato phytochrome coding sequences has been mapped to chromosome 10 (Sharrock *et al.*, 1988), whereas the *au* locus is situated on chromosome 1. This means that either this sequence encodes a phytochrome type different from that absent in the *au* mutant or that the deficiency of spectrophotometrically active phytochrome in the *au* mutant is not a result of a mutation of a structural phytochrome gene *(phy)*.

One aspect of the *au* phenotype is its reduced germination in D compared to wild type, which can be overcome by GA (Koornneef *et al.*, 1985; Georghiou and Kendrick, 1991). Since GA influences seedling growth and development, other conditions have been sought which result in high germination of *au* seed batches in D, if studies are to be carried out on seedling development. Recently, improvement of germination by chilling, high temperature and/or nitrate treatment have been studied (Georghiou and Kendrick, 1991). The freshly harvested seeds, which are dormant, can be induced to germinate after treatment with a combination of chilling and nitrate. Moreover, exposure to continuous R led to an increase in germination of *au*-mutant seed batches, while no inhibitory effect of continuous FR was observed, in contrast to wild type which exhibits a strong FR irradiance-dependent inhibition of germination. Lipucci di Paola *et al.* (1988) have found a promotion of seed germination by FR for *au* mutants and suggested that this is the consequence of the absence of an inhibitory FR-HIR.

At the etiolated seedling stage, the *au* mutant is characterized by a reduction in: (i) hypocotyl growth inhibition in white light (Koornneef *et al.*, 1985), R, B and UV-A (Adamse *et al.*, 1988b; this thesis); (ii) chlorophyll and chloroplast development (Koornneef *et al.*, 1985; Ken-Dror and Horwitz, 1990); (iii) anthocyanin content (this thesis) and (iv) the photoregulation of the transcript levels of chlorophyll *a*/*b*-binding proteins of photosystem I and II, plastocyanin and subunit II of photosystem I (Sharrock *et al.*, 1988; Oelmüller *et al.*, 1989). This pleiotropic phenotype, coupled with lack of phytochrome in etiolated *au*-mutant tissues is precisely that predicted for a photoreceptor mutant and suggests that the lack of phytochrome (presumably PI) plays an important role in these processes.

Adult light-grown plants of both wild type and *au* mutant exhibit a quantitatively similar elongation growth response to end-of-day (EOD) FR treatment (Adamse *et al.*, 1988b; López-Juez *et al.*, 1990b; this thesis) and changes in the R:FR photon ratio (Whitelam and Smith, 1991), indicating the presence of functional phytochrome in light-grown *au*-mutant plants. Although the most plausible inference is to ascribe these responses to PII, which is

predicted to accumulate in the mutant, the identification of the molecular nature of phytochrome detected in light-grown tissues has only just been initiated (López *et al.*, 1991).

1.3.2. Tomato high-pigment mutants

A spontaneous mutant at the high pigment *(hp)* locus was found as early as 1917 (Reynard, 1956). Besides mutants with *hp* characteristics which are allelic with the *hp* mutation *e.g.* the dark-green mutant of Manapal tomato (Mochizuki and Kamimura, 1986), there are also mutants which are similar in some aspects to the mutant phenotype, but are non-allelic with *hp*, such as *hp-2* (Soressi, 1975), *atroviolatia (atv)* (Rick *et al*, 1968) and intensive pigment *(ip)* (Rick 1974) mutants. Furthermore, plants with *hp*-like characteristics at their seedling stage were obtained when high levels of an oat PI gene were expressed in tomato (Boylan and Quail, 1989).

The monogenic recessive *hp* mutants are characterized by features such as dark-green foliage and immature fruit colour due to high chlorophyll levels (Sanders *et al.*, 1975), higher lycopene and carotene content resulting in deeper red fruits (Thompson *et al.*, 1962) and high levels of anthocyanin (Kerr, 1965, von Wettenstein-Knowles, 1968). Mochizuki and Kamimura (1985) observed that *hp*-mutant hypocotyls had more anthocyanin when grown in yellow light and used this as a selection criterion. This method was slightly modified by Dr. M. Koornneef and coworkers (pers. comm.), who use 24 h R to select *hp* mutants. There are also indications of reduced plant height in *hp* mutants: (i) hypocotyl growth was more inhibited than that of wild type when the seedlings were grown in R or yellow light (Mochizuki and Kamimura, 1985); (ii) hypocotyl dry weight was lower than in wild type when the seedlings were grown in white light (von Wettenstein-Knowles, 1968). Thompson *et al.* (1962) reported that the seed germination of *hp* mutants was lower than wild type and that the stems of *hp* mutant plants were more brittle resulting in a higher mortality than wild type. The pleiotropic nature of the *hp* mutant suggests that it has a modification of a basic process affecting plant morphogenesis rather than it being a mutation affecting a specific response.

1.3.3. Tomato *procera* mutant

The *procera (pro)* mutant is a mutant with long internodes in white light and its phenotype is remarkably similar to that of the wild type treated with GA (Jones 1987; Jupe *et al.*, 1988). However, this mutant does not have increased GA levels (Jones 1987). Since the mutant resembles wild-type plants that have been grown under supplementary FR, it was suggested by Adamse *et al.* (1988c) that the *pro* mutant could be similar to the cucumber long-hypocotyl *(lh)* mutant. Therefore it is a possible candidate for a mutant deficient in the function of light-stable phytochrome.

1.3.4. Cucumber long-hypocotyl mutant

The original mutant was found in the progeny of X-ray irradiated *Cucumis* plants of complex hybrid origin (van der Knaap and de Ruiter, 1978) and was

crossed with the cucumber cultivar Stereo (de Ruiter & Zonen BV., Bleiswijk, The Netherlands). Koornneef and van der Knaap (1983) isolated the monogenic recessive mutant on the basis of its elongated hypocotyl in white light and showed that it was allelic with a similar mutant described and named long hypocotyl *(lh)* by Robinson and Shail (1981).

The *lh* mutant and the wild type are similar with respect to: rate and percentage of seed germination, hypocotyl growth rate in D, R/FR reversibility of hypocotyl growth in etiolated seedlings, phytochrome content in seeds and apparent phytochrome synthesis in D which precedes germination (Adamse *et al.*, 1987). Moreover, spectrophotometric measurements of phytochrome *in vivo* (Adamse, 1988) and *in vitro* (Nagatani *et al.*, 1989), along with immunochemical reactivity against the monoclonal antibody mAP5 (Nagatani *et al.*, 1989) indicate that the *lh* mutant and wild type contain a similar amount of phytochrome in etiolated tissue.

De-etiolated seedlings of the *lh*-mutant, compared to wild type, fail to respond to R, show no R/FR reversibility of hypocotyl growth (Adamse *et al.*, 1987), lack the EOD FR response on stem elongation (Adamse *et al.*, 1988a; López-Juez *et al.*, 1990a), show no or severely reduced responses to changes in the R:FR photon ratio (Ballaré *et al.*, 1991; Whitelam and Smith, 1991) and contain 35-40% of the spectrophotometrically detectable phytochrome in wild-type flower petals and Norflurazon-bleached leaves (Adamse *et al.*, 1988a). Based on some of these findings Adamse *et al.* (1988a) proposed that de-etiolated seedlings of the *lh* mutant lack the function attributable to light-stable phytochrome (PII) due to deficiency of the photoreceptor, which accumulates in light-grown seedlings. However, this hypothesis was not confirmed by immunochemical and biochemical investigations by Nagatani *et al.* (1989). These workers demonstrated that young de-etiolated seedlings contain similar amounts of phytochrome in mutant and wild-type extracts. Since in both genotypes about one quarter of the phytochrome could be immunoprecipitated by an antibody which recognizes PI, they tentatively concluded that the wild type and the *lh* mutant had similar PI and PII levels, although there was no positive identification of PII.

Additional information about the mutants will be given in the subsequent chapters of this thesis.

1.4. OUTLINE OF THE THESIS

In this thesis the concept that different members of the phytochrome family have discrete physiological roles is elaborated and an attempt made to assign specific functions to them. Moreover, a possible phytochrome transduction-chain mutant, the *hp* mutant, is characterized. The experiments were performed with tomato seedlings (Chapters 2, 3 and 4), adult light-grown tomato plants (Chapter 5) or cucumber seedlings (Chapter 6).

In Chapter 2 the *au* and *hp* mutants, the *au,hp* double mutant and their isogenic wild type are used to study the influence of the mutations on the photoregulation of anthocyanin synthesis.

The *hp* mutation is characterized in terms of anthocyanin synthesis and hypocotyl growth in Chapter 3.

In Chapter 4 the basis of the *hp*-mutant phenotype is investigated and the quantitative consequences of the *au* and *hp* mutations on two phytochrome-regulated responses are discussed on the basis of fluence rate-response relationships.

Chapter 5 analyses the response of adult light-grown *au*, *hp*, *au,hp* and *pro* tomato mutants and their isogenic wild type to EOD FR treatment, which is a method used to trigger the phytochrome-mediated shade-avoidance-like response.

The results of experiments designed to determine the phytochrome content and hypocotyl growth of *lh*-mutant and wild-type cucumber seedlings are presented in Chapter 6.

Finally, in Chapter 7 the results of Chapters 2 through 6 are integrated and discussed in the light of relevant literature.

1.5. PUBLISHED WORK

The data presented in Chapters 2 through 6 have been published in:

Adamse, P., J.L. Peters, P.A.P.M. Jaspers, A. van Tuinen, M. Koornneef and R.E. Kendrick (1989) Photocontrol of anthocyanin synthesis in tomato seedlings: a genetic approach. Photochem. Photobiol. **50,** 107-111.

Peters, J.L., A. van Tuinen, P. Adamse, R.E. Kendrick and M. Koornneef (1989) High pigment mutants of tomato exhibit high sensitivity for phytochrome action. J. Plant Physiol. **134,** 661-666.

Peters J.L., M.E.L. Schreuder, S.J.W. Verduin and R.E. Kendrick (in press) Anthocyanin content and hypocotyl growth of photomorphogenetic tomato mutants. Photochem. Photobiol.

Peters J.L., M.E.L. Schreuder, G.H. Heeringa, J.C. Wesselius, R.E. Kendrick and M. Koornneef (1992) Analysis of the response of photomorphogenetic tomato mutants to end-of-day far-red light. Acta Hort. **305,** 67-77.

Peters J.L., R.E. Kendrick and H. Mohr (1991) Phytochrome content and hypocotyl growth of long-hypocotyl mutant and wild-type cucumber seedlings during de-etiolation. J. Plant Physiol. **137,** 291-296.

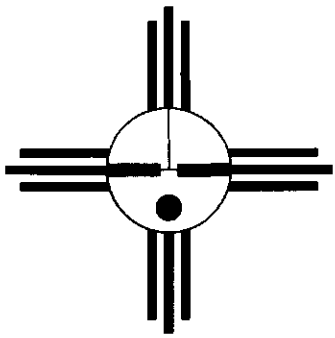
Parts of chapters 1 and 7 have been published in:

López-Juez E., A. Nagatani, W.F. Buurmeijer, J.L. Peters, M. Furuya, R.E. Kendrick and J.C. Wesselius (1990) Response of light-grown wild-type and *aurea*-mutant tomato plants to end-of-day far-red light. J. Photochem. Photobiol., B: Biology **4,** 391-405.

Kendrick, R.E., P. Adamse, E. López-Juez, M. Koornneef, J.L. Peters and J.C. Wesselius (1991) The significance of mutants in phytochrome research. In: Photobiology: The science and its applications (Edited by E. Riklis). Plenum Press pp.437-444.

Peters J.L., J.C. Wesselius, K.C. Georghiou, R.E. Kendrick, A. van Tuinen and M. Koornneef (1991) The physiology of photomorphogenetic tomato mutants. In: Phytochrome properties and biological action (Edited by B. Thomas and C.B. Johnson). Springer Verlag, Heidelberg pp.237-247.

Koornneef M., A. van Tuinen, L.H.J. Kerckhoffs, J.L. Peters and R.E. Kendrick (in press) Photomorphogenetic mutants of higher plants. In: Proc. of the 14th Int. Conf. on Plant Growth Substances (Edited by C.M. Karssen, L.C. van Loon and D. Vreugdenhil) Kluwer Academic Publ., Dordrecht, The Netherlands.



CHAPTER 2

PHOTOCONTROL OF ANTHOCYANIN SYNTHESIS IN TOMATO SEEDLINGS: A GENETIC APPROACH

Abstract. The photocontrol of anthocyanin synthesis in dark-grown seedlings of tomato (*Lycopersicon esculentum* Mill.) has been studied in: an *aurea (au)* mutant which is deficient in the labile type of phytochrome, a high pigment *(hp)* mutant which has the wild-type level of phytochrome and the double mutant *au,hp*, as well as the wild type. The *hp* mutant demonstrates phytochrome control of anthocyanin synthesis in response to a single red light (R) pulse, whereas there is no measurable response in the wild type and *au* mutant. After pretreatment with 12 h blue light (B) the phytochrome regulation of anthocyanin synthesis is 8-fold higher in the *hp* mutant than in the wild type, whilst no anthocyanin is detectable in the *au* mutant, thus suggesting that it is the labile pool of phytochrome which regulates anthocyanin synthesis. The *au,hp* double mutant exhibits a small (3% of that in the *hp* mutant) R/far-red light (FR) reversible regulation of anthocyanin synthesis following a B pretreatment. It is proposed that the *hp* mutant is hypersensitive to the FR-absorbing form of phytochrome (Pfr) and that this hypersensitivity establishes response to the low level of Pfr, which is below detection limits in phytochrome assays, in the *au,hp* double mutant.

2.1. INTRODUCTION

In response to short irradiations (minutes) with red light (R) small amounts of anthocyanin are formed in dark (D)-grown seedlings of several species, *e.g.* cabbage, rye (Mancinelli, 1984a, b; Mancinelli and Rabino, 1985) and mustard (Lange *et al.*, 1971). The effect of a single R pulse can be reversed by a far-red light (FR) pulse applied immediately afterwards, indicating phytochrome control. The extent of the R/FR reversible response is small when compared to the extent of the response to prolonged irradiations (Mancinelli, 1985). However, no inductive response (to a single R pulse) on anthocyanin production has been observed in D-grown seedlings of tomato, sorghum and turnip

(Drumm and Mohr, 1978; Mohr and Drumm-Herrel, 1981; Drumm-Herrel and Mohr, 1982a; Mancinelli and Schwartz, 1984; Mancinelli, 1985; Drumm-Herrel, 1987). A light pretreatment is required before an inductive response, working *via* phytochrome, can be observed in these seedlings. The magnitude of the R/FR reversible response depends upon: (i) the duration and spectral quality of the pretreatment; (ii) the duration of the D interval between the end of the pretreatment and the light pulses; (iii) seedling age (Mancinelli, 1985). Pretreatments with blue light (B) and UV are particularly effective and have been interpreted as increasing the responsiveness to the FR-absorbing form of phytochrome (Pfr) due to light absorption by a B/UV photoreceptor (Drumm-Herrel and Mohr, 1982a; Oelmüller and Mohr, 1984). In tomato it appears that the interaction between the B/UV photoreceptor and phytochrome is not obligatory, since pretreatment with either R or FR, absorbed by phytochrome alone is also effective. When continuous irradiation was applied to induce anthocyanin formation in seedlings without an inductive pulse at the end of the light treatment, B, UV-A and UV-B exerted a strong response, whereas FR and R were only slightly effective (Drumm-Herrel and Mohr, 1982a).

Whereas short irradiations can induce the synthesis of a small amount of anthocyanin in some species, the synthesis of large quantities in these, as well as other species, requires prolonged exposures to high fluence rates. Such requirements are typical of a so-called 'high irradiance response' (HIR) (Downs, 1964; Hartmann, 1966; Lange *et al.*, 1971; Mancinelli, 1985). Mancinelli (1983) distinguishes between three main groups of HIRs for anthocyanin synthesis on the basis of spectral sensitivity. Group I: B/UV, R and FR are all effective. Group II: B/UV and R are active; the effect of FR is negligible. Group III: The B/UV region is the only active region. In seedlings of tomato and rye maximum action is in the B/UV region as in group III, but R and FR are also effective, although much less than B and UV (Drumm-Herrel and Mohr, 1982a, b; Mancinelli and Schwartz, 1984). It is difficult to explain the effectiveness of B in HIRs on the basis of phytochrome alone. Results of experiments based on the 'light-equivalence principle' (Chapter 1) have indicated that a B/UV photoreceptor is also involved in the photoregulation of anthocyanin synthesis by B in tomato seedlings (Sponga *et al.*, 1986).

The experiments described in this chapter were designed to achieve a better understanding of the photocontrol of anthocyanin synthesis in tomato seedlings by utilizing a mutant with an enhanced capacity for anthocyanin synthesis, the high pigment *(hp)* mutant. Increased responsiveness of the *hp* mutant towards light was inferred from the observation that in contrast to wild type, it forms high levels of anthocyanin and is reduced in height when grown under broad-band R or yellow light (Mochizuki and Kamimura, 1985). In addition, the *aurea (au)* mutant, which is deficient in the light-labile type of phytochrome (PI) (Koornneef *et al.*, 1985; Parks *et al.*, 1987; Adamse *et al.*, 1988b), the *au,hp* double mutant and their wild type were studied.

2.2. MATERIALS AND METHODS

2.2.1. Plant material

The isolation of the long-hypocotyl *au* mutant from wild-type tomato (*Lycopersicon esculentum* Mill.) cv. Moneymaker has been described by Koornneef *et al.* (1981, 1985). This mutant has been identified as a photoreceptor mutant: the amount of phytochrome in etiolated seedlings being <5% of that detected in the wild type (Koornneef *et al.*, 1985; Parks *et al.*, 1987). The *hp* monogenic recessive mutant is characterized by mature fruits that have a higher lycopene and carotene content; darker green foliage and immature fruit colour, than that of the wild type (Thompson *et al.* 1962). Plants of this genotype, which was isolated as a spontaneous mutant as early as 1917, also produce higher levels of anthocyanin, although this depends on the growing conditions (von Wettstein-Knowles, 1968). The important observations in this respect are that the hypocotyl just below the soil surface has a pinkish colour in the mutant (Kerr, 1965) and that there is a high anthocyanin content in hypocotyls of seedlings when grown under continuous R (Mochizuki and Kamimura, 1985). The *hp* mutant used in the present experiments was derived from a cross and one subsequent backcross of the original mutant, named Webb Special or Black Queen (received from C.M. Rick, Davis, USA; collection number LA279) with the cv. Moneymaker. In addition a *hp* mutant in the genetic background of the cv. Ailsa Craig was used with its isogenic wild type, which were obtained from J.W. Maxon Smith, Glasshouse Crop Research Institute, Littlehampton, UK, where the Ailsa Craig *hp* mutant was obtained by repeated backcrossings of the original mutant with this cultivar (Maxon Smith and Ritchie, 1983). The *au,hp* double mutant was isolated from the progeny derived from a cross of the Moneymaker *au* mutant with the Webb Special *hp* mutant according to the procedure outlined in Fig. 2.1, which allowed the isolation of the double mutant without prediction of its phenotype. The overall morphology of this double mutant resembled the *au* phenotype.

2.2.2. Anthocyanin assay

Seeds were surface sterilized for 5 min in a 1% (vol/vol) Na-hypochlorite solution (commercial bleach), washed for 30 min in running tap water and sown in transparent plastic boxes (10x10x8 cm) on 10 layers of filter paper saturated with distilled water. After incubation for 84 h in D at 25°C the irradiation schedule was started. All handling of the plants prior to extraction took place under a dim green safelight. Each treatment was terminated with a 24 h D period, after which anthocyanin extraction was carried out. Samples of 10 seedlings of uniform height were taken from each box and extracted with 1.2 ml acidified (1% HCl, wt/vol) methanol for 48 h in D with shaking. A Folch partitioning (Folch *et al.*, 1957) was performed by adding 0.9 ml H_2O and 2.4 ml chloroform to the extracts and centrifugation for 30 min at 4800 rpm. The absorbance of the top phase was determined with an Aminco DW-2a spectrophotometer at 535 nm. In most experiments 4 replicates were carried

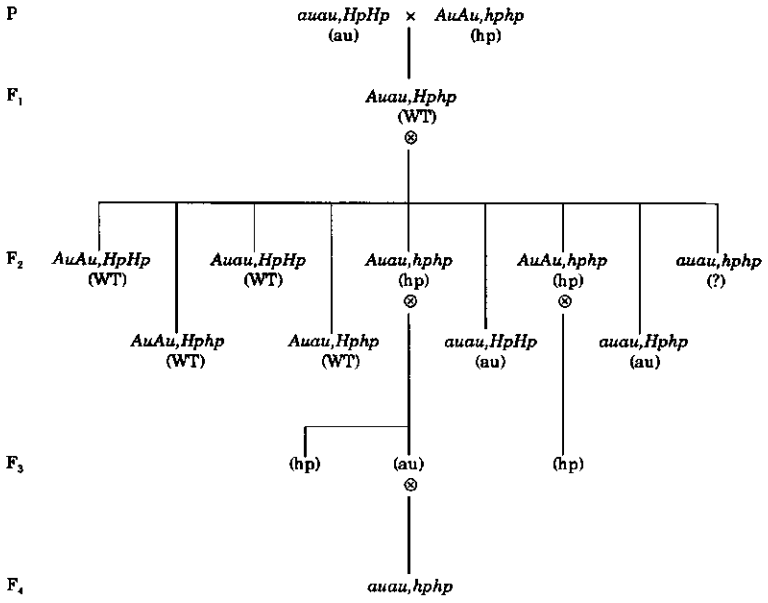


Figure 2.1. Scheme describing the isolation of the *au,hp* double mutant of tomato without an *a priori* prediction of its phenotype. Between brackets description of the phenotype and conditions used for selection: WT = wild-type seedlings with almost white hypocotyls after 24 h low-irradiance red light (R) and short hypocotyls in white light; au = no visible anthocyanin after 24 h low-irradiance R, long hypocotyls and reduced chlorophyll content in white light; hp = etiolated seedlings with red hypocotyls after 24 h low-irradiance R and short hypocotyls in white light. (× = crossed; ⊗ = selfed). See text for further explanation.

out for each treatment and the results represent the means ± S.E. of the results from two or more independent experiments.

2.2.3. Light sources

The broad-band light sources for B, R and FR are described in detail by Koornneef *et al.* (1980). The fluence rate of the B, used as pretreatment, and of the R and FR pulses was 2.1 μmol m^{-2} s^{-1} ± 5%.

2.3. RESULTS AND DISCUSSION

The Ailsa Craig *hp* mutant exhibits a small, yet significant induction of anthocyanin synthesis in a 24 h D period in response to a single R pulse at 84 or 90 h from sowing (Fig. 2.2). The isogenic wild type shows no measurable response to a single R pulse, confirming earlier observations with tomato (Drumm-Herrel and Mohr 1982a; Mancinelli and Schwartz, 1984). The effect of

R on the *hp* mutant is reversed by FR, indicating phytochrome control. Two pulses of R, the first after 84 h D and the second after an additional 6 h D, were much more effective in the induction of anthocyanin synthesis than one R pulse in the *hp* mutant (Fig. 2.2). Even with two R pulses, the wild type shows no significant induction of anthocyanin synthesis. When a R pulse is followed immediately by a FR pulse this results in a low photoequilibrium (φ = Pfr/Pr+Pfr, where Pr is the R-absorbing form of phytochrome). Despite the low φ-value as a result of the R/FR pulses at 84 h, a dramatic enhancement of the response to a R pulse at 90 h is observed (Fig. 2.2). This indicates that the response of the *hp* mutant to the first pulse is extremely sensitive to Pfr. A FR pulse instead of R/FR pulses at 84 h leads to the same enhancement to a R pulse at 90 h (data not shown). The fact that the effect of R pulses are not fully reversible by FR in these experiments (Fig. 2.2) is an indication that the *hp* mutant exhibits high sensitivity to Pfr. On the basis of the model proposed by Oelmüller and Mohr (1985) this can be explained as follows: phytochrome controls the anthocyanin synthesis *via* two different routes. Firstly, as the effector of the terminal response it sets in motion the signal-transduction chain which eventually leads to the appearance of anthocyanin. Secondly, it determines the effectiveness of Pfr in mediating anthocyanin synthesis. In

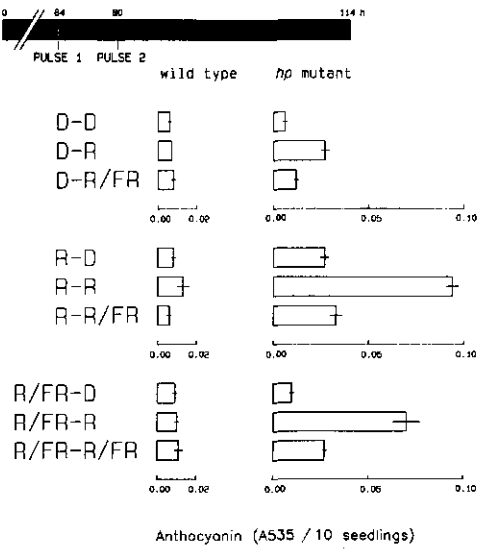


Figure 2.2. Anthocyanin content of tomato seedlings of the Ailsa Craig *hp* mutant and its isogenic wild type following one or two inductive light pulses. The first pulse was given after 84 h incubation in darkness (D) and the second pulse after a further 6 h in D. Pulses consisted of red light (R; 5 min, 2.1 μmol m^{-2} s^{-1}), R followed immediately by far-red light (FR; 15 min, 2.1 μmol m^{-2} s^{-1}) (R/FR) and were given in all possible combinations. After the time of the second pulse all treatments were terminated with a 24 h D period before anthocyanin extraction.

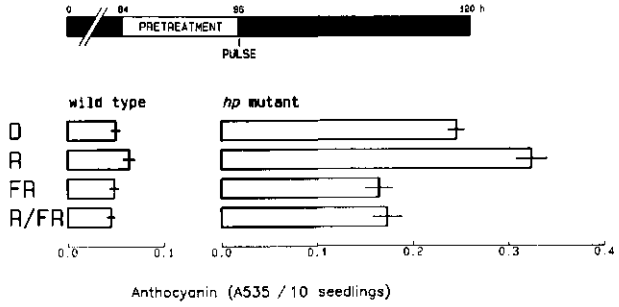


Figure 2.3. Anthocyanin content of tomato seedlings of the Ailsa Craig *hp* mutant and its isogenic wild type after a 12 h blue light (B) pretreatment (2.1 μmol m^{-2} s^{-1}) terminated with: no light pulses (D), a pulse of red light (R; 5 min, 2.1 μmol m^{-2} s^{-1}), a pulse of far-red light (FR; 15 min, 2.1 μmol m^{-2} s^{-1}) or R followed by FR (R/FR). After a further 24 h darkness anthocyanins were extracted.

other words, it determines the responsiveness of the seedling for anthocyanin synthesis as a result of production of Pfr. This responsiveness can also be established by a pretreatment with B or UV.

The experiments presented here are compatible with this hypothesis: the first R pulse is proposed to increase the sensitivity to Pfr, resulting in an enhanced response to the second R pulse. A D period longer than 6 h resulted in a reduced response (data not shown). Apparently the increased responsiveness to a second pulse is gradually lost if the D period is too long. This is similar to the observation of Mohr and Drumm-Herrel (1983), who showed that the amplification of sensitivity to Pfr induced by UV-B for anthocyanin synthesis of wheat coleoptiles was completely lost if a 12 h D period separated the UV-B treatment and the inductive R pulse.

These results indicate that the conclusion of Drumm-Herrel and Mohr (1982a) and Mancinelli and Schwartz (1984), that single light pulses have no effect on anthocyanin synthesis in the tomato hypocotyl, should be reformulated to state that single light pulses have no measurable effect on anthocyanin synthesis. Only in the case of the *hp* mutant is response to such treatments clearly detectable due to its enhanced capacity for anthocyanin synthesis.

After a 12 h B pretreatment, anthocyanin was produced in both the Ailsa Craig *hp* mutant and the isogenic wild type (Fig. 2.3). A pulse of R terminating the B pretreatment resulted in a stimulation of anthocyanin synthesis and a pulse of FR or R followed by FR reduced anthocyanin synthesis compared to seedlings which received a 12 h B treatment without following light pulses. However, the response of the *hp* mutant was much larger than the wild type. Similar results were obtained with the Moneymaker wild type and *hp* mutant (Fig. 2.4). The *au* mutant had practically no anthocyanin after these treatments. Without pretreatment with B no anthocyanin could be detected in the double mutant *(au,hp)* (data not shown). However, after a B pretreatment this mutant showed a small (less than wild type), yet significant, R/FR

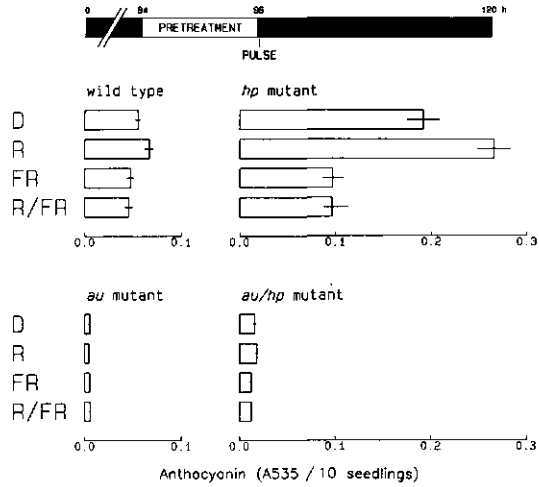


Figure 2.4. Anthocyanin content of tomato seedlings of the Moneymaker *au*, *hp*, *au,hp* and wild type after a 12-h blue light (B) pretreatment (2.1 μmol m^{-2} s^{-1}) terminated with: no light pulses (D), a pulse of red light (R; 5 min, 2.1 μmol m^{-2} s^{-1}), a pulse of far-red light (FR; 15 min, 2.1 μmol m^{-2} s^{-1}) or R followed by FR (R/FR). After a further 24-h darkness anthocyanins were extracted.

reversible response (Fig. 2.4). Apparently the phytochrome which is below detection limits in the *au* mutant is now sufficient to induce a low level of anthocyanin synthesis due to the increased sensitivity as a result of the *hp* mutation.

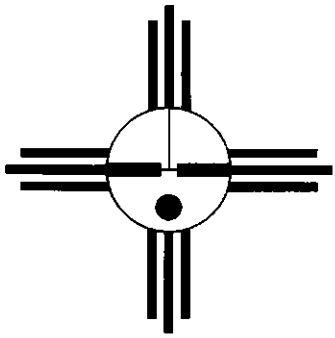
The amount of phytochrome-regulated, *i.e.* the R/FR reversible, anthocyanin synthesis in the *hp* mutant, produced in 24 h D after a B pretreatment, is amplified about 8-fold compared to the wild type. Taking the anthocyanin synthesis in the *hp* mutant after B pretreatment as 100%, by extrapolation, the response after a single R pulse in the case of the wild type would be expected to be about 1%. This would correspond to a difference in absorbance of about $2\ 10^{-3}$, which is below detection limits. This explains why no effect of a single R pulse on anthocyanin synthesis in tomato hypocotyls has previously been observed. The anthocyanin synthesis in the *au,hp* double mutant after a B pretreatment is about 3% of the maximum response. The spectrophotometrically and immunologically detectable phytochrome level in the *au* mutant is <5% of that in the wild type (Parks *et al.*, 1987) and the spectrophotometrically detectable phytochrome level in the *hp* mutant and wild type are similar (Adamse, 1988). In other words, the absence of at least 95% of PI results in only 3% of the anthocyanin synthesis under phytochrome control in the *hp* mutant after B pretreatment. This clearly suggests that it is the 'bulk' light-labile phytochrome pool in etiolated seedlings which regulates anthocyanin synthesis at this stage. Provisional observations indicate that some anthocyanin is formed in hypocotyls of older seedlings of the *au* mutant

grown in white light. It is possible that this is a response to the light-stable phytochrome type (PII) that accumulates under such conditions (Pratt and Cordonnier, 1987).

Apart from enhancing the inductive, R/FR reversible anthocyanin synthesis, light pretreatments also influence the subsequent response to prolonged irradiations; they cause a reduction in the photosensitivity of the HIR and a change in spectral sensitivity (Mancinelli and Schwartz, 1984). It has been suggested that this difference in sensitivity between D- and light-grown seedlings is the consequence of changes in the relative amount of PI and PII, as indicated by results obtained in studies of the effectiveness of intermittent light treatments on HIR anthocyanin synthesis (Mancinelli and Rabino, 1985). Phytochrome action in D-grown seedlings might be mainly due to PI which is lost rather rapidly by destruction and would have only a limited time to act. In light-grown seedlings a larger proportion of phytochrome action might be exercised by PII which would have an extended time to act. This is consistent with the observation, that the differences in effectiveness between intermittent light treatments with short and long D intervals between successive irradiations are more pronounced in D-grown seedlings than in light-grown ones, where action would be expected mainly due to PI and PII, respectively (Mancinelli and Rabino, 1985).

The cause of the enhanced anthocyanin synthesis in the *hp* mutant is still unclear. Preliminary spectrophotometric measurements of phytochrome in D-grown seedlings indicated no differences in phytochrome content between wild type and *hp* mutant (Adamse, 1988) and therefore the difference does not appear to be due to a higher [Pfr]. Since the *hp* phenotype is pleiotropic (high chlorophyll, carotenoid and ascorbic acid content of the fruits, in addition to the enhanced anthocyanin synthesis in seedlings) it is possible that the increased anthocyanin synthesis is just one of a number of processes stimulated due to an apparent increased sensitivity to Pfr. Earlier work by von Wettstein-Knowles (1968) and Mochizuki and Kamimura (1985) showed that the *hp* mutant had a lower hypocotyl dry weight than the wild type when grown in the light. This points to increased inhibition of the hypocotyl growth by light, compatible with higher sensitivity to Pfr in the *hp* mutant than in the wild type. However, whatever the final explanation turns out to be for this 'amplified' response, the *hp* mutant is a useful tool for study of the photocontrol of anthocyanin synthesis.

Acknowledgements. We thank C.J. Hanhart for assistance and M.T.M. Sprik for conducting preliminary experiments; Prof. C.M. Rick for seeds of LA279 and Dr. J.W. Maxon Smith for seeds of the *hp* mutant in the Ailsa Craig background.



CHAPTER 3

PHYSIOLOGICAL CHARACTERIZATION OF HIGH-PIGMENT MUTANTS OF TOMATO

Abstract. Anthocyanin synthesis and hypocotyl growth have been studied in tomato (*Lycopersicon esculentum* Mill.) seedlings of high-pigment *(hp)* mutants, *aurea (au)* mutants deficient in the light-labile type of phytochrome, an *au,hp* double mutant and wild types. The phytochrome-controlled (red light (R) / far-red light reversible) anthocyanin synthesis occurring in a 24 h dark (D) period after a 12 h pretreatment with R or blue light (B) is similar in the *hp* mutant, whereas in wild type, pretreatment with B is more effective than R. When grown under continuous broad-band UV-A, B and R for 5 d the *au* and *au,hp* double mutants only accumulate low levels of anthocyanin compared to the wild type and the *hp* mutant. Under these conditions the *hp* mutant accumulates the highest levels of anthocyanin, but the relative effectiveness of R and B in the *hp* mutant and the wild type is reversed (B being more effective than R in wild type, whereas in the *hp* mutant R is more effective than B). These results suggest that the *hp* mutation enables maximum anthocyanin synthesis to be achieved without activation of the B photoreceptor. When grown for 7 d in a regime of 14 h white light / 10 h D the activation of anthocyanin synthesis is reduced in the *au* and *au,hp* mutants compared to wild type and the *hp* mutant. After the same treatment the hypocotyls of *hp*-mutant seedlings of each cultivar are similar in length to those of their corresponding wild types, while those of the *au* and *au,hp* mutants are longer. However, after 5 d continuous low fluence-rate (3 μmol m^{-2} s^{-1}) UV-A, B and R the hypocotyl growth of the *hp* mutant is inhibited more than wild type. The *au,hp* double mutant shows a small, but significantly higher anthocyanin accumulation, as well as increased inhibition of hypocotyl growth than the *au* mutant. It is proposed that the *hp* mutation increases the sensitivity to the light-labile phytochrome pool.

3.1. INTRODUCTION

The monogenic recessive high-pigment *(hp)* mutants of tomato are characterized by mature fruits that have a higher lycopene and carotene content, increased levels of ascorbic acid, immature fruit colour and darker green foliage than their isogenic wild types (Thompson *et al.*, 1962). The dark-green foliage and fruit colour are due to high chlorophyll levels (Sanders *et al.*, 1975). A spontaneous mutant at the *hp* locus was found as early as 1917

(Reynard, 1956). A recessive mutant with more extreme dark-green fruits was discovered in plantings of 'Manapal' tomato (Konsler, 1973) and was initially called dark green *(dg)*. Subsequently this mutant was shown to be allelic with *hp* (Mochizuki and Kamimura, 1986). Under appropriate conditions *hp* mutants exhibit high levels of anthocyanin (von Wettenstein-Knowles, 1968). Important in this respect is the observation that the hypocotyls just below the soil surface have a pinkish colour (Kerr, 1965) and that there is a higher anthocyanin content in hypocotyls of *hp* seedlings compared to wild type when grown under continuous red light (R) or yellow light (Mochizuki and Kamimura, 1985). There are also indications of reduced plant height in *hp* mutants: (i) hypocotyl growth was more inhibited than that of wild type when the seedlings were grown in R or yellow light (Mochizuki and Kamimura, 1985); (ii) hypocotyl dry weight was lower than in wild type when the seedlings were grown in white light (von Wettenstein-Knowles, 1968). Thompson *et al.* (1962) reported that the seed germination of *hp* mutants was lower than wild type and that the stems of *hp*-mutant plants were more brittle, resulting in a higher mortality than wild type.

The pleiotropic nature of the *hp* mutant suggests that it has a modification of a basic process affecting plant morphogenesis rather than it being a mutation affecting a specific response. In the previous chapter it was suggested that increased sensitivity to the far-red light (FR)-absorbing form of phytochrome (Pfr) is the cause of the increase in photoregulation of anthocyanin synthesis in the *hp* mutant. The experiments described in this chapter were conducted to further test this hypothesis.

3.2. MATERIALS AND METHODS

3.2.1. Plant Material

Two long-hypocotyl mutants *(au^w* and *au)*, four *hp* mutants and the *au,hp* double mutant of *Lycopersicon esculentum* Mill. in different genetic backgrounds were used and are listed in Table 3.1.

The isolation of the long-hypocotyl *au* mutant after mutagenesis of the wild-type tomato (cv. Moneymaker) has been described by Koornneef *et al.* (1981, 1985). The Moneymaker *hp* mutant used here was derived from a cross and one subsequent backcross of the original mutant, Webb Special, with the cv. Moneymaker. The *au,hp* double mutant was isolated from the progeny derived from a cross of the Moneymaker *au* mutant with the Webb Special *hp* mutant according to the procedure described in Chapter 1, by first selecting plants homogenous for *hp* in the F_2 progeny of this cross and subsequently selecting *au*-type plants in the F_3 progeny of selected *hp,hp* plants that were heterozygous for *au*.

3.2.2. Light pulses, light pretreatments and genetic experiments

In the experiments with short R pulses, 12 h R or blue light (B) pretreatment

Table 3.1. Genetic stocks used in the present study.

Mutant allele	Genetic background	Collection number	Reference
au^{w}	Moneymaker	W616	Koornneef *et al.*, 1985
au	Ailsa Craig	GCR 360	Maxon Smith and Ritchie, 1983
hp*	Ailsa Craig	GCR 60	Maxon Smith and Ritchie, 1983
hp*	Moneymaker		Chapter 1
hp*	Webb Special	LA 279	Reynard, 1956
hp^{w}	GT**	WB3	This Chapter
au,hp	Moneymaker/Webb Special		Chapter 1

* Stocks derived from the same *hp* (Webb Special) allele.
** GT is a Tobacco Mosaic Virus resistant breeding line of the Moneymaker type kindly provided by De Ruiter & Zonen BV., Bleiswijk, The Netherlands.

and for the genetic tests the seeds were surface sterilized for 5 min in a 1% (vol/vol) Na-hypochlorite solution (commercial bleach), washed for 30 min in running tap water and sown in transparent plastic boxes (10x10x8 cm) on 10 layers of filter paper saturated with distilled water. In the short R-pulse experiments the anthocyanin content of seedlings was determined 112 h after sowing at 25°C. Dark (D) controls were maintained in D throughout and the treatment consisted of a 5 min R pulse at 84 h or 5 min R pulses at 84 h and 90 h from sowing. In the experiment with R or B pretreatment the seedlings were exposed to the following irradiation schedule: 84 h D / 12 h R or B pretreatment terminated with: (i) no pulse; (ii) a 5 min R pulse or (iii) a 5 min R pulse followed by a 10 min FR pulse / 24 h D. At the end of both experiments the anthocyanin content (10 seedlings per assay) was determined. For the genetic tests the colour of the hypocotyls of different wild-type and *hp* parents and the F_2 progeny of wild-type x *hp*-mutant crosses was scored visually after a 96 h D / 24 h R / 24 h D treatment.

3.2.3. White light experiments

Seeds were sown in 40x60 cm plastic trays filled with potting compost. The trays were placed in a 14 h white light (36 W m^{-2}) / 10 h D regime at 22°C. From the time that the hypocotyl hook was 0.5 to 1.0 cm above the surface, 20 plants were sampled each day for 7 d for determination of hypocotyl length and anthocyanin content (5 seedlings per assay). The length of each hypocotyl was measured with a ruler.

3.2.4. Continuous broad-band light experiments

Seeds were sown in 13x13 cm square plastic pots filled with potting compost and incubated in D for 80 h *(au* and *au,hp)* or 60 h (wild type and *hp)* at 25°C. The irradiation with continuous UV-A, B and R (3 μmol m^{-2} s^{-1}) was started just before the seedlings emerged through the surface. The length of each hypocotyl was measured daily for 5 d with a ruler under dim green safe light. At the end

of the experiment 20 seedlings were used for anthocyanin determination (5 seedlings per assay). In addition the hypocotyl length of plants grown in complete D for the duration of the experiment was measured.

3.2.5. Light Sources

The cabinets used for R and B pretreatments were previously described by Joustra (1970). The R was provided by 11 Philips TL40/103339 tubes in combination with a 3 mm plexiglas filter (Röhm and Haas; nr. 501) and B by 2 mercury lamps (Tungsram IMP HGMI 400/C) with a 10 cm water filter and a 3 mm plexiglas filter (nr. 248). To reduce the fluence rate of R without affecting its quality a perforated aluminium sheet was used. White light was obtained from a bank of fluorescent tubes (Philips TLF 40W/33). The broad-band UV-A, B, R and FR, used for pulse and continuous irradiation, were the same as those described by Koornneef *et al.* (1980). The actual fluence rates and irradiation times used are given in the table and figure legends.

3.2.6. Anthocyanin assay

Samples of 5 or 10 seedlings or one leaf (adult plants) were taken and extracted with 1.2 ml acidified (1% HCl, wt/vol) methanol for 48 h in D with shaking. A Folch partitioning (Folch *et al.*, 1957) was performed after adding 0.9 ml H_2O and 2.4 ml chloroform to the extracts and centrifugation for 30 min at 4800 rpm. The absorbance of the top phase was determined with an Aminco DW-2a spectrophotometer at 535 nm (A535). In most experiments the results represent the mean ± SE of 4 replicates for each treatment and are usually the means of two or more independent duplicate experiments.

Table 3.2. The colour of tomato hypocotyls of parents and F_2 progeny of two wild type (WT) × mutant crosses and a mutant × mutant cross after 96 h dark (D) / 24 h red light (8 μmol m^{-2} s^{-1}) / 24 h D.

Genotype (genetic background)		Number of hypocotyls	
		White	Red
Wild type	(Moneymaker)	30	0
	(Ailsa Craig)	34	0
	(GT)	30	0
hp mutant	(Moneymaker)	0	40
	(Ailsa Craig)	0	30
	(Webb Special)	0	55
hp^w mutant	(GT)	0	35
F_2 WT (Moneymaker) × *hp* (Webb Special)		183	58*
F_2 WT (GT) × hp^w (GT)		165	53*
F_2 *hp* (Webb Special) × hp^w (GT)		0	28

* No significant deviation from an expected segregation of 3 white : 1 red hypocotyl as tested with a χ^2 test (P>0.05).

3.2.7. Dual-wavelength assay of phytochrome

For measurements of the phytochrome content of 3 to 4 d old etiolated seedlings a custom-built dual-wavelength spectrophotometer was used with the measuring beam set at 730 nm and the reference beam at 800 nm (Spruit, 1970). In the case of the *au,hp* double mutant the seeds were germinated on filter paper moistened with a 10^{-5} M mixture of the gibberellins $(GA)_4$ and GA_7. This treatment has been shown to improve the germination without having any influence on the phytochrome level detected spectrophotometrically (Koornneef *et al.*, 1981) The photoreversible difference in absorbance difference $\Delta(\Delta A730\text{-}800$ nm) following saturating alternate actinic R and FR irradiation is proportional to the total phytochrome present. The actinic light consisted of the light from a 250 W quartz-iodide projection lamp filtered through interference filters (Balzer B40 type, Balzer, Liechtenstein) with peak transmission at 660 and 730 nm and 10 nm half band width at 50% of the transmission maximum.

3.3. RESULTS AND DISCUSSION

3.3.1. Genetics

The seedling test of screening for *hp* mutants by examination of seedlings grown for 10 d under yellow vinyl film (Mochizuki and Kamimura, 1986) was modified by irradiating 3 d old seedlings germinated in D for only 24 h with R and screening for seedlings with red hypocotyls (*i.e.* containing anthocyanin) after a subsequent 24 h D period. Using this procedure *hp* mutants could be unambiguously distinguished from non-*hp* phenotypes and if necessary transplanted into soil. Selfed progenies of hybrids of the *hp* mutant with wild type showed the 3:1 ratio expected for a monogenic recessive trait (Table 3.2). In cases where 'red' seedlings from segregating populations were grown to maturity they had the dark-green immature fruit colour, darker green foliage and slightly reduced height compared to wild type, characteristics of the *hp* mutant. In a selfed progeny of a plant raised from ethylmethanesulphonate (EMS)-treated seeds of the genotype GT, a mutant (collection number WB3 [hp^w]) was isolated with a similar, but more extreme phenotype, having an even more pronounced reduced plant height, darker green leaves and immature fruit colour, than the Webb Special *hp* mutant. This mutant phenotype also segregated as monogenic recessive in the seedling test and was allelic to *hp* (Table 3.2). Allelism was inferred both from the dark-green phenotype of the hybrid of hp^w and the original *hp* mutant and the absence of wild-type segregants in selfed progeny of this hybrid. The observation that the different *hp* characteristics segregate as one trait and occur in mutants that have arisen independently indicates that these characteristics are all controlled by a mutation in the same *(Hp)* gene and therefore should have a similar biochemical and physiological basis.

3.3.2. Anthocyanin synthesis

Contrary to wild type, which exhibits no detectable response, the *hp* mutant demonstrates phytochrome control of anthocyanin synthesis in response to a single R pulse (Chapter 2). All *hp* mutants used show a quantitatively similar response to one or two R pulses (Table 3.3). It can therefore be concluded that the response of the *hp* mutants to R pulse(s) is independent of the *hp* allele and genetic background.

After pretreatment with 12 h B the phytochrome-regulated, R/FR reversible, anthocyanin synthesis during a subsequent 24 h D period is approximately 4 to 8-fold higher in the *hp* mutant than in the wild type (Fig. 3.1 and Chapter 2). The R/FR-reversible anthocyanin synthesis is similar after pretreatment with R and B in the *hp* mutant, whereas in the wild type, pretreatment with B is about 3 times more effective than R (Fig. 3.1). The *hp* mutation appears to increase the sensitivity to Pfr and enables maximum anthocyanin synthesis to be achieved without activation of the B photoreceptor.

The *hp* mutant exhibits a lower level of anthocyanin after 5 d continuous B than after 5 d continuous R or UV-A (Fig. 3.2). This may be explained by the fact that B leads to a maintenance of approximately 45% of total phytochrome as Pfr at equilibrium, while R and UV-A both lead to approximately 80% (Mancinelli, 1986). The results indicate that, despite the increased sensitivity to Pfr of the *hp* mutant, anthocyanin synthesis is not saturated by the Pfr level maintained by B. However, in the wild type, B and UV-A induces the highest level of anthocyanin synthesis, because unlike the *hp* mutant, the wild type requires the activation of the B/UV-A photoreceptor to exhibit maximal anthocyanin synthesis (Drumm-Herrel and Mohr, 1982b). The *au* (Koornneef *et al.*, 1985) and *au,hp* mutants are deficient in the light-labile phytochrome type (PI) (in 3 d old seedlings at a stage when phytochrome is readily detectable in wild type no phytochrome could be detected spectrophotometrically in the *au,hp* double mutant) and have a very low level of anthocyanin after 5 d monochromatic light, while the *hp* mutant shows an enhanced anthocyanin content compared to wild type (Fig. 3.2). These results suggest the importance of the PI pool in the regulation of anthocyanin synthesis. It also indicates that the *hp*

Table 3.3. Anthocyanin content (10^3 A535 / 10 seedlings ± SE) of *hp*-mutant and wild-type (WT) tomato seedlings (cv. Ailsa Craig, Moneymaker, GT and Webb Special) after 112 h darkness (D) or 112 h D interrupted by a 5 min R pulse (3 μmol m^{-2} s^{-1}) at 84 h or 5 min R pulses at 84 h and 90 h (2xR).

Pulse	Ailsa Craig		Moneymaker		GT		Webb Special
	WT	*hp*	WT	*hp*	WT	hp^w	*hp*
Dark	4±1	4±1	13±5	7± 1	10±1	7± 1	6± 0
R	5±1	16±2	6±1	19± 2	9±1	24± 3	15± 2
2xR	8±1	76±7	5±1	109±14	13±1	110±11	96±14

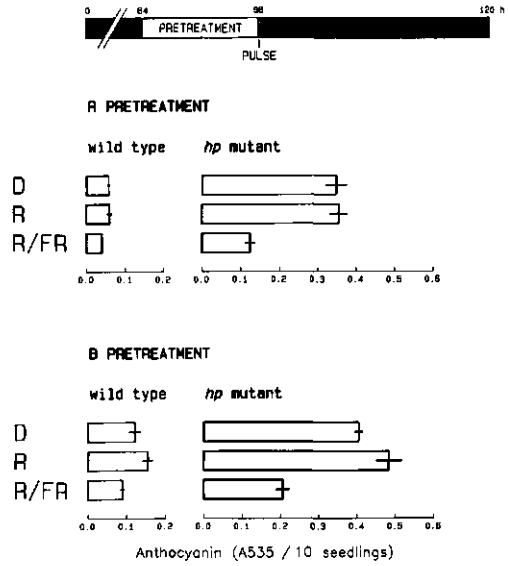


Figure 3.1. Anthocyanin content of *hp*-mutant and wild-type tomato seedlings (cv. Ailsa Craig) after a 12 h red-light (R) or blue-light (B) pretreatment (25 μmol m^{-2} s^{-1}) terminated with: no pulse (D), a 5 min pulse of R (8 μmol m^{-2} s^{-1}) (R) or a 5 min pulse of R followed by a 10 min pulse of far-red (3 μmol m^{-2} s^{-1}) (R/FR).

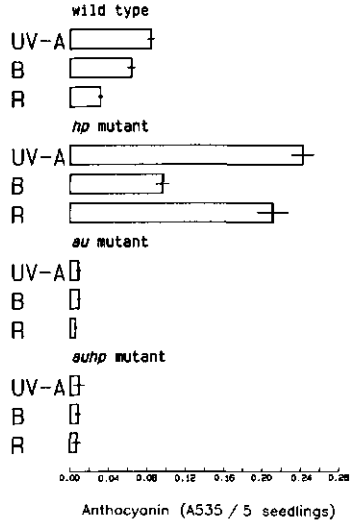


Figure 3.2. The anthocyanin content of *au*-, *hp*-, *au,hp*-mutant and wild-type tomato seedlings (cv. Moneymaker) after 5 d in continuous UV-A, blue light (B) or red light (R) (3 μmol m^{-2} s^{-1}).

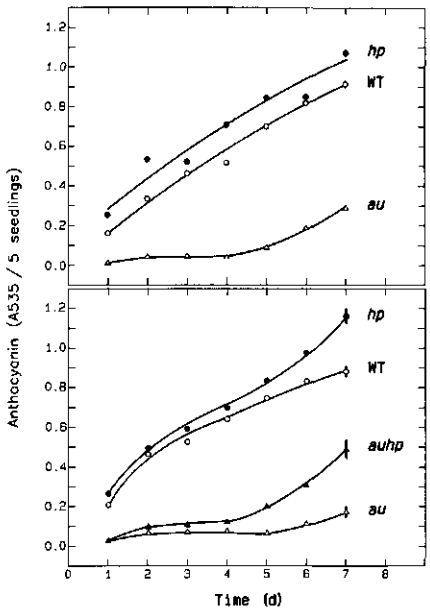


Figure 3.3. The anthocyanin content of tomato seedlings of (i) Top: *au*, *hp* and isogenic wild type (WT) (cv. Ailsa Craig); (ii) Bottom: *hp* (Webb Special), *au,hp* (Moneymaker / Webb Special), *au* and WT (cv. Moneymaker) grown in a 14 h white light (36 W m^{-2}) / 10 h dark regime for 7 d. For clarity only the SE for the last data points are shown, others were all <5% of the mean.

mutation does not result in the constitutive expression of anthocyanin synthesis genes.

Daily measurements of the anthocyanin content of tomato seedlings of different genotypes grown for 7 d in a regime of 14 h white light / 10 h D are shown in Fig. 3.3. The response of the *au* and the *au,hp* double mutant is reduced compared to wild type and *hp* mutant which both show an immediate increase in anthocyanin synthesis (Fig. 3.3). This reduction is probably a consequence of the lack of PI, in the *au* and *au,hp* mutants. The increase in anthocyanin level in the *au* mutant after 4 to 5 d is intriguing. This response appears to be hypocotyl specific and associated with aging. Possible explanations include: (i) that an independent B photoreceptor-controlled process operates at this stage; (ii) that after several days the B photoreceptor induces enhanced sensitivity to the very low level of Pfr present; (iii) that a different type of phytochrome, such as the light-stable type of phytochrome (PII) that accumulates in light-grown tissues (Pratt and Cordonnier, 1987), eventually becomes functional in the regulation of anthocyanin synthesis. However, leaves of light-grown adult *au* plants have very low anthocyanin levels compared to wild type (0.16 ± 0.08 and 4.40 ± 0.40 A535 / g fresh weight, respectively). This observation suggests that the PI pool is also functional in the regulation of anthocyanin synthesis in light-grown plants.

Table 3.4. Hypocotyl length of tomato seedlings after 7 d in a regime of 14 h white light (36 W m^{-2}) / 10 h dark at 22°C.

Genotype	Genetic background	Hypocotyl length ± SE (cm)
Wild type	Ailsa Craig	2.56 ± 0.03
	Moneymaker	3.07 ± 0.04
au mutant	Ailsa Craig	5.19 ± 0.11
	Moneymaker	6.82 ± 0.12
hp mutant	Ailsa Craig	2.28 ± 0.03
	Webb Special	2.93 ± 0.04
au,hp mutant	Moneymaker/Webb Special	4.95 ± 0.08

3.3.3. Hypocotyl growth

If the *hp* mutant is hypersensitive to Pfr then other phytochrome-controlled processes such as inhibition of hypocotyl growth should show a higher photosensitivity than wild type. Work by von Wettstein-Knowles (1968) and Mochizuki and Kamimura (1985) provided the first indication that this is the case. After growth for 7 d in a regime of 14 h white light (36 W m^{-2}) / 10 h D the hypocotyls of *hp* seedlings of each cultivar are similar in length to those of their corresponding wild type (Table 3.4). The B component of the white light is probably sufficient to induce saturation and maximum inhibition occurs. The *au*-mutant hypocotyls are clearly longer than their respective wild types and the *au,hp* mutant has hypocotyls intermediate between the lengths of the corresponding monogenic mutants. However, in low fluence rate monochromatic UV-A, B and R the hypocotyl growth of the *hp* mutant is significantly more inhibited than that of the wild type (Fig. 3.4). The *au* mutant and the *au,hp* double mutant, which both are deficient in PI, are hardly inhibited by R.

The cause of the differences between the *hp* mutant and wild type is still unclear. The phytochrome content of comparable samples of 3 d old etiolated wild-type and *hp*-mutant seedlings was 7.98 ± 0.17 and 6.56 ± 0.50 10^3 Δ(ΔA)730-800 nm ± SE, respectively. Therefore the differences observed cannot be explained by a higher absolute [Pfr]. In Chapter 2 it was suggested that the *hp* mutant is hypersensitive to Pfr. The two characteristics of the *hp* mutants studied here: reduced plant height under low fluence-rate monochromatic light and enhanced anthocyanin synthesis, and the observation that the *au,hp* double mutant exhibits a small response to the low level of Pfr present (Chapter 2; Figs. 3.3, 3.4) are all observations compatible with this hypothesis. This situation is similar to the *Phycomyces madH* mutants which are hypersensitive with respect to light induced phototropic behaviour (Galland and Lipson, 1985).

Enhanced anthocyanin synthesis and the reduction in elongation growth are characteristics opposite to that of the *au* phenotype. Other processes where phytochrome plays a role during development are chlorophyll synthesis and germination. Both processes are reduced in the *au* mutant compared to the wild

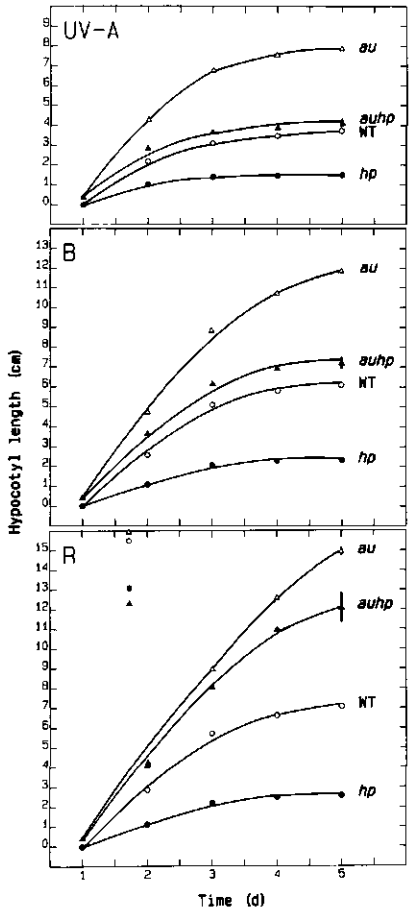


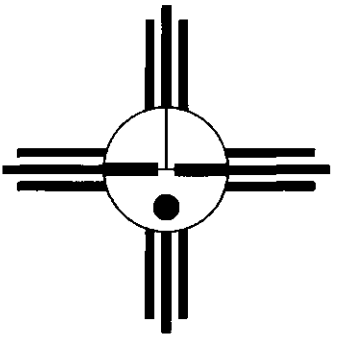
Figure 3.4. The hypocotyl length of *au*-, *hp*-, *au,hp*-mutant and wild-type (WT) (cv. Moneymaker) tomato seedlings grown in continuous UV-A, blue light (B) and red light (R) (3 μmol m^{-2} s^{-1}) for 5 d. The mean hypocotyl length ± SE of the dark controls of *au*, *hp*, *au,hp* mutants and wild type at the end of the 5 d period was 15.9 ± 0.1, 13.1 ± 0.1, 12.3 ± 0.6 and 15.5 ± 0.1 cm, respectively, and is indicated by the appropriate symbol in the lower figure. For clarity only the SE for the last data points are shown, others are all <5% of the mean.

type (Koornneef *et al.*,1985). The *hp* mutant with its dark-green foliage and immature fruit colour has higher chlorophyll levels than wild type (Sanders *et al.*, 1975). The rate and percentage of germination of non-*hp* tomato cultivars have been reported to be significantly higher than those of the *hp* lines (Thompson *et al.*, 1962). In our experiments, germination of the *hp* mutants was very similar to wild types.

In conclusion, the *hp* mutation appears to be pleiotropic for phytochrome-controlled responses and is therefore not simply an anthocyanin response

mutant. De-etiolation of tomato seedlings is the result of co-action between phytochrome and the B-photoreceptor systems and it has been proposed that activation of the B photoreceptor enhances the sensitivity to Pfr (Mohr, 1986). The *hp* mutation mimics B treatment, enabling complete de-etiolation to occur under R, whereas wild type requires co-action between the B photoreceptor and phytochrome. Since most recessive mutants are loss-of-function mutants, the increase in responsiveness to Pfr can be considered as a release of a constraining process (inhibitory process). Such a process could be a component of the transduction chain between the B photoreceptor and phytochrome. Although the biochemistry of the underlying processes is still unknown, the identification of a mutant which has high sensitivity to Pfr facilitates the study of phytochrome-controlled responses during de-etiolation.

Acknowledgements. We thank C.J. Hanhart and P.A.P.M. Verhoeven-Jaspers for technical assistance, Prof. C.M. Rick for seeds of Webb Special, J.W. Maxon Smith for seeds of the *au* and *hp* mutant in the Ailsa Craig background and De Ruiter & Zonen BV., Bleiswijk for the seeds of GT.



CHAPTER 4

FURTHER PHYSIOLOGICAL CHARACTERIZATION OF A HIGH-PIGMENT MUTANT OF TOMATO

Abstract. A high-pigment *(hp)* mutant, which shows exaggerated phytochrome responses and three other genotypes of *Lycopersicon esculentum* Mill. cv. Ailsa Craig: the *aurea (au)* mutant deficient in the bulk light-labile phytochrome (PI) pool, the *au,hp* double mutant and their isogenic wild type were used in this study. Measurements of phytochrome destruction in red light (R) revealed that the exaggerated responses of the *hp* mutant are not caused by a higher absolute phytochrome level or a reduced rate of phytochrome destruction. Fluence-response relationships for anthocyanin synthesis after a blue-light pretreatment were studied to test if the *hp* mutant conveys hypersensitivity to the far-red light (FR)-absorbing form of phytochrome (Pfr), *i.e.* the threshold of Pfr required to initiate the response is lower. However, the response range for the *hp* mutant and wild type was identical, although the former exhibited a 6-fold larger response. Moreover, the kinetics of anthocyanin accumulation in continuous R were similar in the wild-type and *hp*-mutant seedlings, despite the latter accumulating 9-fold more anthocyanin. Since the properties of phytochrome are the same, the *hp* mutation appears to affect the state of responsiveness amplification, *i.e.* the same amount of Pfr leads to a higher response in the *hp* mutant. We therefore propose that the *hp* mutation is associated with an amplification step in the phytochrome transduction chain. Escape experiments showed that the anthocyanin synthesis after different light pretreatments terminated with a R pulse was still 50% FR reversible after 4 to 6 h darkness, indicating that the Pfr pool regulating this response must be relatively stable. However, fluence-rate response relationships for anthocyanin synthesis and hypocotyl growth induced by a 24 h irradiation with 451, 539, 649, 693, 704 and 729 nm light showed no or a severely reduced response in the *au* and *au,hp* mutants, suggesting the importance of PI in these responses. We therefore propose that the capacity for anthocyanin synthesis (state of responsiveness amplification) could be established by PI, while the anthocyanin synthesis is actually photoregulated *via* a stable Pfr pool. The *Hp*-gene product is proposed to be an inhibitor of the state of responsiveness amplification for responses controlled by this relative stable Pfr species.

4.1. INTRODUCTION

The control of plant development by light, photomorphogenesis, involves at least three different groups of photoreceptors: phytochrome, a blue light

(B)/UV-A photoreceptor and a UV-B photoreceptor (Mohr, 1986). Moreover, several different types of phytochrome are known (for review see Furuya, 1989). Current data for pea (Abe *et al.*, 1989), *Arabidopsis* (Sharrock and Quail, 1989) and oat (Pratt *et al.* 1991) suggest that these different phytochrome types are the products of different genes. Work of Sharrock *et al.* (1988) and Hauser and Pratt (1991) suggest that there are also multiple phytochrome genes present in tomato. Since the different genes and corresponding products are unknown for tomato, we will use the terms light-labile (PI) and light-stable (PII) phytochrome for the two pools of phytochrome which have been identified at the spectrophotometric and physiological level.

The analysis of mutants is a valuable means of understanding photomorphogenesis. This is shown by studies of the *aurea (au)* mutant of tomato, which is deficient in the bulk PI pool (for review see Peters *et al.*, 1991b). The monogenic recessive high pigment *(hp)* mutant shows characteristics opposite to the *au* phenotype: seedlings have an increased anthocyanin content (Chapters 2 and 3) and a reduced hypocotyl length in red light (R), B, UV-A (Chapter 3) and yellow light (Mochizuki and Kamimura, 1985) compared to the wild type. Moreover, in light-grown plants the chlorophyll content is particularly high in immature fruit tissues (Sanders *et al.*, 1975) and mature fruits have a higher lycopene and carotenoid content than wild type (Kerr, 1965). Unlike wild type, the *hp* mutant does not require the activation of the B photoreceptor to exhibit high levels of anthocyanin synthesis and attains complete de-etiolation under R alone (Chapter 3). Since etiolated *au*-mutant and *au,hp*-double mutant seedlings, both deficient in the bulk PI pool, show no or only a small (3% compared to *hp*) R/FR reversible anthocyanin response after a B pretreatment (Chapter 2), respectively, it has been suggested that a PI pool regulates anthocyanin synthesis at the seedling stage and that the *hp* mutation does not result in the constitutive expression of genes involved in anthocyanin synthesis. Possible explanations for the *hp* phenotype compared to the wild type are: (1) a higher absolute phytochrome content; (2) a higher stability of the active FR-absorbing form of phytochrome (Pfr); (3) hypersensitivity to Pfr, *i.e.* the threshold of Pfr required to initiate the response is lower or (4) responsiveness amplification, *i.e.* the same amount of Pfr leads to a higher response (Mohr, 1986).

We have tested these four hypotheses and report on the quantitative consequences of the *au* and the *hp* mutations on two phytochrome-regulated responses: anthocyanin synthesis and hypocotyl growth.

4.2. MATERIALS AND METHODS

4.2.1. Plant material

Four genotypes of tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) were used: the *au* mutant, the *hp* mutant, the *au,hp* double mutant and their isogenic wild type. The original *au* and *hp* mutants were obtained from Dr.

J.W. Maxon Smith, Glasshouse Crop Research Institute, Littlehampton, UK (Maxon Smith and Ritchie, 1983) and the seeds used were propagated in Wageningen (Genetics Department, Wageningen Agricultural University, The Netherlands). The *au,hp* double mutant was isolated as described in Chapter 2. The seeds were surface sterilized for 10 min in a 1% (vol/vol) Na-hypochlorite solution (commercial bleach), washed for 5 min in running tap water and sown in plastic boxes with transparant lids (10x10x7 cm) on one layer of filter paper (T300-45mm, Schut BV., Heelsum, The Netherlands) at 25°C. The filter paper was moistened with 1.8 ml of a solution containing 10 mM Na/K phosphate buffer (pH 7.5) and 5 mM KNO_3. For the phytochrome determination the seeds were sown on 5 layers of filter paper (nr. 5 Ederol-9 cm, Binzer, Hatzfeld, Germany) moistened with 7.2 ml of the germination buffer mentioned above. Under these conditions a high proportion of the seed populations germinated, even the *au* mutants which tend to germinate poorly in distilled water (Georghiou and Kendrick, 1991).

4.2.2. Phytochrome determination

The phytochrome content of 5 d old seedlings was measured using a custom-built dual-wavelength spectrophotometer with the measuring beam set at 730 nm and the reference beam at 667 nm (Spruit, 1970). The photoreversible difference in absorbance difference $\Delta(\Delta A667\text{-}730)$ following saturating alternate actinic R and FR irradiation is proportional to the total phytochrome (Ptot) present. Total phytochrome is Pfr plus the R-absorbing form of phytochrome (Pr). The actinic light consisted of the light from a 250 W quartz-iodide projection lamp filtered through interference filters (Balzers, Liechtenstein) with peak transmission at 661 and 734 nm and ± 10 nm band width at 50% of the transmission maximum (λ_{max}).

4.2.3. Anthocyanin assay

Samples of 10 seedlings were taken and extracted with 1.2 ml acidified (1% HCl, wt/vol) methanol for 24 h in dark (D) while being shaken. A partitioning was performed after adding 0.9 ml H_2O and 2.4 ml chloroform to the extracts and centrifugating for 30 min at 2000 g (after Folch *et al.* 1957). The absorbance of the top phase was determined with a Beckman DU-64 spectrophotometer at 535 nm (A535).

4.2.4. Hypocotyl length measurement

The hypocotyl length was measured under a dim green safelight (0.8 μmol m^{-2} s^{-1}) to the nearest 0.5 mm with a ruler before the light treatment and afterwards in daylight prior to the anthocyanin extraction.

4.2.5. Light sources

The cabinets used for broad-band R, B and FR were described previously by Joustra (1970). The R was obtained from Philips TL40/103339 tubes in combination with a 3 mm red plexiglas filter (Röhm and Haas nr. 501, Darmstadt, Germany). The λ_{max} was at 658 nm; band width at 50% of λ_{max}

14 nm; fluence rate 82.0 μmol m^{-2} s^{-1} and the phytochrome photoequilibrium (φ), Pfr/Ptot, was 0.88. The B (λ_{max} at 442 nm; band width at 50% of λ_{max} 82 nm; fluence rate 5.0 μmol m^{-2} s^{-1}; φ = 0.44) was obtained from Philips TL 40/18 tubes in combination with a 3 mm blue plexiglass filter (Röhm and Haas nr. 248). Broad-band FR (fluence rate 20 μmol m^{-2} s^{-1}; φ = 0.03) was obtained by filtering the light of Philips 60 W tungsten filament lamps through one layer of blue and one layer of red 3 mm plexiglass filter (Röhm and Haas nr. 627 and 501, respectively). The fluence rate of this light regime was calculated by substracting the fraction transmitted by a 780 nm cut-off filter (Schott, Mainz, Germany) from the total fluence rate.

Fluence (rate)-response relationships were studied using a threshold-box unit. A series of neutral beam-splitters (100x145x1 mm, reflection/transmission: 50/50%, Balzers, Liechtenstein) enabled 7 fluence rates to be studied simultanuously over a fluence-rate range of 3 log units. The plastic boxes containing the seedlings were placed on height-adjustable platforms, which rotated during irradiation to optimize the light distribution. As light sources a Philips 250/7748S quartz-iodide projection lamp or a xenon-arc lamp (Osram XBO-4000 W/HS 0FR) were used in combination with the following interference filters of λ_{max}: 451 nm (Balzers, band width at 50% of λ_{max} 13.8 nm, φ = 0.44); 539 nm (Baird-Atomic, Bedford, USA, band width at 50% of λ_{max} 10.5 nm, φ = 0.68); 649 nm (Baird-Atomic, band width at 50% of λ_{max} 11.6 nm, φ = 0.88); 693 nm (Baird-Atomic, band width at 50% of λ_{max} 18.8 nm, φ = 0.56); 704 nm (Baird-Atomic, band width at 50% of λ_{max} 15.1 nm, φ = 0.16); 729 nm (Baird-Atomic, band width at 50% of λ_{max} 11.5 nm, φ = 0.02).

The fluence rate was measured with a photodiode meter (Optometer type 80X and Optometer model 370, United Detector Technology Inc., Hawthorne, USA) and spectral quality monitored with a spectral analyzer (Rofin-Sinar Laser Ltd., Weybridge, UK) coupled with a microcomputer. The φ values were calculated on the basis of the extinction coefficients for Pr and Pfr and quantum yield data for Pr and Pfr reported by Lagarias *et al.* (1987).

Table 4.1. The fluences of red light (660 nm) and the calculated Pfr/Ptot produced in the different compartments of the threshold box.

Compartment	Irradiation time (s)	Fluence (μmol m^{-2})	Pfr/Ptot
1	300	2698	0.88
1	140	1259	0.88
2	140	479	0.85
3	140	191	0.65
4	140	79.4	0.36
5	140	32.4	0.18
6	140	10.7	0.09
7	140	3.47	0.05

4.2.6. Experiments

The kinetics of phytochrome destruction in 5 d old *hp*-mutant and wild-type plants were determined by exposing the seedlings to broad-band R at 25°C.

To study the fluence-response relationship, seedlings, which were grown in D for 84 h, were given a 12 h broad-band B pretreatment terminated with a 20 min saturating broad-band FR pulse (φ = 0.03). In the threshold box these seedlings were then exposed to different fluence rates of R (projector lamp in combination with a 666 nm interference filter, Baird-Atomic, band width at 50% of λ_{max} 16 nm) for 140 or 300 s, which resulted in the calculated proportions of Pfr/Ptot given in Table 4.1. The formulae of Mancinelli (1986) and the extinction coefficients and quantum yield data reported by Lagarias *et al.* (1987) were used to calculate Pfr/Ptot (Table 4.1). The anthocyanin content was measured 24 h after the R pulse.

To determine the kinetics of anthocyanin accumulation under continuous R, seedlings were grown in D for 89 h and then transferred to continuous broad-band R. The data presented are for one experiment, which on repetition gave qualitatively the same result.

For the escape from FR reversibility experiments 84 h old D-grown seedlings were given the following pretreatments terminated with a 10 min saturating broad-band R pulse (φ = 0.88): (i) 12 h B; (ii) 10 min broad-band R, 6 h D; (iii) 20 min broad-band FR, 6 h D. At different times after the terminating, inductive R pulse 20 min broad-band FR was given (φ = 0.03). The anthocyanin content was measured 24 h after the inductive R pulse.

The fluence rate-response relationships for anthocyanin synthesis and hypocotyl growth were determined in the threshold box in combination with light sources and interference filters (λ_{max} = 451, 539, 649, 693, 704, 729 nm). The seedlings were grown in D for 89 h (wild type and *hp* mutant) or 102 h (*au* and *au,hp* mutants, to compensate for their delay in germination) before exposure to 24 h continuous light. Anthocyanins were extracted at the end of the light treatment after hypocotyl length measurement.

4.3. RESULTS AND DISCUSSION

4.3.1. Phytochrome content and destruction kinetics

The *hp*-mutant phenotype is clearly pleiotropic and opposite to that of the *au* mutant, exhibiting exaggerated phytochrome responses with regard to induction of anthocyanin synthesis and inhibition of hypocotyl growth during de-etiolation. Since the *au* mutant exhibits reduced responses compared to the wild type as a consequence of phytochrome deficiency (Adamse *et al.*, 1988b) and exaggerated phytochrome responses have been observed in tomato seedlings overexpressing a *phyA* gene from oat (Boylan and Quail, 1989), possible explanations for the *hp* phenotype are: that etiolated seedlings contain more phytochrome and/or that the phytochrome present is more stable as Pfr, than in the wild type. An experiment in which 5 d old D-grown *hp*-mutant and

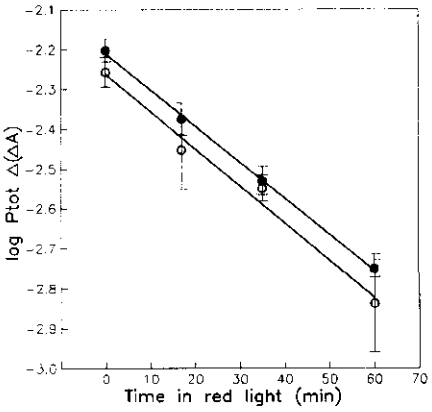


Figure 4.1. Destruction kinetics of total phytochrome (Ptot) ± SE (2 replicates, 3 experiments) in wild-type (o) and *hp*-mutant (•) tomato seedlings after the onset of red light (R) at 25°C. Before the onset of R, at time zero, the plants were kept in darkness for 5 d.

wild-type seedlings were exposed to R to induce a depletion of the PI pool, shows that neither of these explanations is correct. The phytochrome level was similar in the *hp* mutant and wild type and the rate of phytochrome destruction after the onset of R was a first order reaction with a half life of about 32 min in both wild type and *hp* mutant at 25°C (Fig. 4.1).

4.3.2. Anthocyanin synthesis

4.3.2.1. Fluence-response relationship. An alternative explanation for the *hp* phenotype is that it conveys hypersensitivity to Pfr. If this is the case then the fluence-response relationship for phytochrome induction of anthocyanin accumulation would be predicted to be shifted to a lower fluence-response range

Table 4.2. The anthocyanin content ± SE of 3.5 d old dark (D)-grown seedlings treated with a pulse of red light (R) or R followed by far-red light (FR) and the R/FR reversible response after different pretreatments. Light regime: 84 h D; pretreatment; 10 min R; (20 min FR); 24 h D. B = blue light.

Genotype	Pretreatment	Anthocyanin content (A535 / 10 seedlings)		R-R/FR
		R	R/FR	
Wild type	12 h B	0.182 ± 0.008	0.098 ± 0.003	0.084
hp mutant	12 h B	0.768 ± 0.018	0.271 ± 0.005	0.497
hp mutant	10 min R - 6 h D	0.240 ± 0.012	0.073 ± 0.003	0.167
hp mutant	20 min FR - 6 h D	0.107 ± 0.006	0.016 ± 0.001	0.091

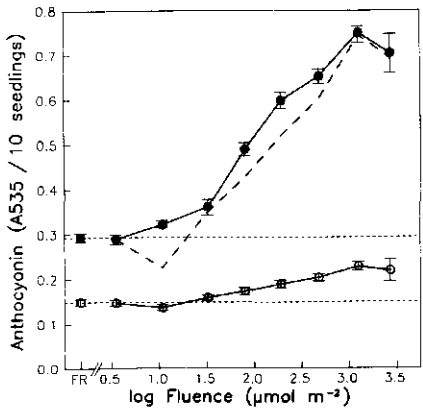


Figure 4.2. Fluence-response relationship for anthocyanin synthesis ± SE (2 replicates, 8 experiments), during a 24 h dark period, induced by a red light (R) pulse in wild-type (○) and *hp*-mutant (●) tomato seedlings. The seedlings were pretreated with 12 h blue light (B) terminated with a saturating far-red light (FR) pulse. The fine dashed lines represent the anthocyanin level after the FR pulse, while the dashed line represents the wild-type data plotted on an amplified scale.

in the *hp* mutant than in the wild type. To test this, 3.5 d old D-grown seedlings were exposed to a 12 h B pretreatment terminated with a saturating FR pulse. The seedlings were then exposed to different fluences of R, which were calculated to result in the Pfr/Ptot values given in Table 4.1. Figure 4.2 shows the fluence-dependent increase in anthocyanin content in the *hp* mutant and the wild type 24 h after such a treatment. The response was saturated at about 1000 μmol m^{-2}, which is the calculated value required for attainment of photoequilibrium (Table 4.1). The R/FR reversible response at photoequilibrium was about 6x greater in the *hp* mutant than in the wild type (Fig. 4.2 and Table 4.2). The wild-type R/FR reversible data plotted on an amplified scale (6x) show that the response range for the *hp* mutant and wild type is identical. In other words, the threshold of Pfr required to initiate the response in the *hp* mutant is not lower when compared to wild type (*i.e.* the mutant is not hypersensitive to Pfr), but the same amount of Pfr leads to a higher response in the *hp* mutant (*i.e.* the mutant exhibits responsiveness amplification).

The explanation for the *hp* phenotype on the basis of hypersensitivity to Pfr was thought unlikely, since the *hp* mutant exhibits such a large R/FR reversible response (Fig. 4.2 and Table 4.2), a feature inconsistent with hypersensitivity to Pfr, where the level of Pfr maintained by FR would be anticipated to lead to an appreciable response.

4.3.2.2. Kinetics. Synthesis of anthocyanin started about 7 h after the exposure of D-grown wild-type and *hp*-mutant seedlings to continuous R and the final level of anthocyanin attained in the *hp* mutant was about 9x that of the wild

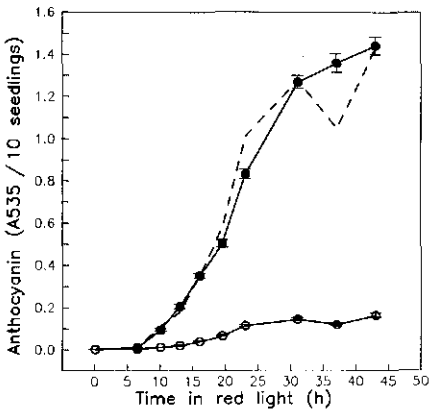


Figure 4.3. The anthocyanin synthesis ± SE (4 replicates, 1 experiment) in wild-type (○) and *hp*-mutant (●) tomato seedlings after different periods of irradiation with red light (R) at 25°C. Before the onset of R the plants were kept in dark for 89 h. The dashed line represents the wild-type data plotted on an amplified scale.

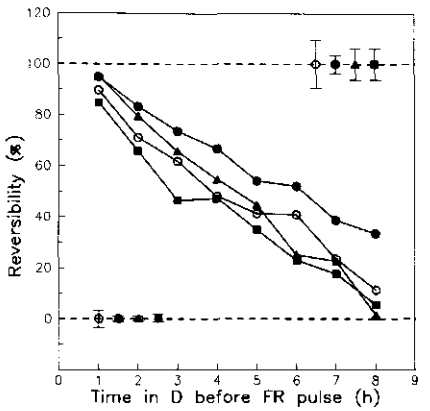


Figure 4.4. The effect of a 20 min far-red light (FR) pulse at different times after a saturating red light (R) pulse on the FR reversibility of anthocyanin synthesis ± SE (4 replicates, 3 experiments), during a 24 h dark (D) period. The tomato wild-type (○) and *hp*-mutant (●) seedlings were pretreated with 12 h blue light (B) and the *hp* mutant was pretreated with a FR pulse + 6 h D (▲) or a R pulse + 6 h D (■). Before the light pretreatment, the plants were kept in D for 84 h. The R/FR reversibility is expressed as % of the maximum R/FR reversible response, *i.e.* the anthocyanin synthesis after a R pulse minus that after a R pulse immediately followed by a FR pulse. Absolute levels of anthocyanin corresponding to 0 and 100% are given in Table 4.2.

type (Fig. 4.3). However, the kinetics of anthocyanin accumulation are almost identical, indicating that whatever process or mechanism is involved (presumably the photoregulation of genes in the flavonoid biosynthetic pathway set in motion by R *via* phytochrome), it appears to be identical in both the *hp* mutant and wild type. The results presented in Figures 4.2 and 4.3 suggest that the *hp* mutation affects the state of responsiveness amplification (Mohr, 1986). To investigate the properties of the phytochrome species involved, escape from FR reversibility was tested.

4.3.2.3. Escape from FR reversibility. The response after a R pulse, preceded by different pretreatments, can be reversed by a subsequent FR pulse (Table 4.2). By increasing the D period between the inductive R and the reverting FR pulse the kinetics of the loss of photoreversibility, which is a reflection of the kinetics of Pfr action, can be studied. The pretreatments: (i) 12 h B and (ii) a R pulse plus 6 h D are anticipated to deplete the bulk PI pool more than the pretreatment with a FR pulse plus 6 h D. Although after a 12 h B pretreatment the R/FR reversible response (R-R/FR) for the *hp* mutant was about 6x greater than in the case of wild type (Table 4.2), the response was still 50% reversible by FR after 5.9 h and 4.4 h D in the *hp* mutant and wild type, respectively (Fig. 4.4). After a pretreatment with a R pulse plus 6 h D the R/FR reversible response was twice that after a pretreatment with a FR pulse plus 6 h D in the *hp* mutant (Table 4.2); the response was still 50% reversible by FR after 3.8 h and 4.3 h D, respectively (Fig. 4.4). From these results there is no indication that the kinetics of Pfr action are more rapid in the *hp* mutant. The interesting conclusion that can be drawn is that the Pfr pool regulating anthocyanin biosynthesis must be more stable than Pfr in the bulk PI pool (Fig. 4.1), since at the time of 50% reversibility, less Pfr would be predicted (on the basis of a calculated Pfr half life of 29 min at 25°C) to be present in the PI pool than is maintained at photoequilibrium by the reverting saturating FR pulse. The escape time for reversibility of the end-of-day FR response on anthocyanin biosynthesis in light-grown tomato plants (Chapter 5) has very similar kinetics to those reported here for seedlings.

4.3.2.4. Fluence rate-response relationships. To study the quantitative effects of the *au* and *hp* mutations fluence rate-response relationships for anthocyanin synthesis and hypocotyl growth during a 24 h continuous light treatment were determined (Figs. 4.5 and 4.6). The *au* and the *au,hp* mutants, which lack at least 95% of the bulk PI pool, show no significant anthocyanin accumulation or hypocotyl growth inhibition during this 24 h light treatment at most wavelengths studied, suggesting the importance of the PI pool for this response. The only exceptions are the fluence rate-response relationships for 451 nm light, implicating the involvement of another photoreceptor apart from phytochrome at this wavelength (Figs. 4.5 and 4.6). For both hypocotyl growth and anthocyanin synthesis it is well known that the B photoreceptor plays an important role (*e.g.* Gaba and Black, 1987; Mancinelli *et al.*, 1991). Moreover, B-uninhibited *(blu)* mutants in *Arabidopsis*, which lack the B-dependent

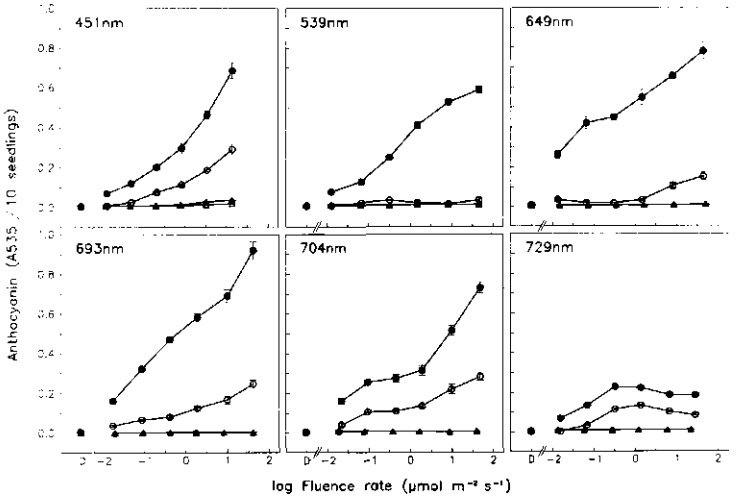


Figure 4.5. Fluence rate-response relationships for the anthocyanin synthesis ± SE (2 replicates, 3 experiments) induced by 24 h irradiation with 451 nm, 539 nm, 649 nm, 693 nm, 704 nm and 729 nm light for wild-type (○), *au*- (△), *hp*- (●) and *au,hp*- (▲) mutant tomato seedlings. D = darkness.

inhibition of hypocotyl growth, but are still inhibited by FR, demonstrate that two photoreceptor systems function in this high-irradiance response (HIR) (Liscum and Hangarter, 1991). However, we can not rule out the possibility that activation of the B photoreceptor results in sensitization to the 'low' residual phytochrome pool in the *au* and *au,hp* mutants. The demonstration of a small R/FR reversible response on anthocyanin biosynthesis in the *au,hp* double mutant after B pretreatment supports this possibility (Chapter 2), as do the results of Oelmüller and Kendrick (1991) on the photoregulation of the expression of four nuclear genes coding for plastidic proteins in the *au* mutant. Although the *au* mutant is lethal under R, it survives under white light. Under long-term B, in contrast to long-term R, the phytochrome in the mutant becomes almost as effective as phytochrome in wild-type seedlings and Oelmüller and Kendrick (1991) proposed that the B component is responsible for the normal gene expression and survival *via* sensitization to residual phytochrome. If the PI pool has evolved specifically to regulate de-etiolation, a mutant missing the entire PI pool would be lethal, suggesting that the low residual phytochrome pool is a PI pool, unless another phytochrome type (*e.g.* PII) takes over its function.

Under 649, 693 and 704 nm light the fluence rate-response relationships for anthocyanin synthesis are complex and appear to consist of at least two components. At moderate to high fluence rates (1-100 μmol m^{-2} s^{-1}) phytochrome will attain photoequilibrium rapidly at the beginning of the 24 h irradiation period (Frankland, 1986). At lower fluences the proportion of phytochrome

maintained as Pfr will be affected by the rate of the non-photochemical processes: Pr synthesis, Pfr destruction and D reversion. As a consequence Pfr/Ptot maintained below 1 μmol m^{-2} s^{-1} is dependent on fluence rate (Heim and Schäfer, 1982; Frankland, 1986). It is proposed that the first part of the 649, 693 and 704 nm fluence rate-response relationships represents a low fluence response (LFR) component, reflecting the gradual attainment of φ, and that the subsequent rise in response is a HIR component. For the *hp* mutant these two components are less clear under 649 and 693 nm than under 704 nm light (Fig. 4.5). This may be caused by the higher φ-value attained at equilibrium under 649 and 693 nm light (0.88 and 0.56 respectively, compared to 0.16 under 704 nm light), which leads to a higher LFR component (Fig. 4.2) and consequently more overlap with the HIR component. The fact that the difference between the anthocyanin synthesis of the wild type and *hp* mutant is much greater at low fluence rates (below 1 μmol m^{-2} s^{-1}) under 649 and 693 nm than under 704 nm light indicates that the LFR component is strongly amplified in the *hp* mutant. Under 539 nm light it is predicted that the HIR component would be very low, since this wavelength is relatively inefficient at phytochrome cycling. Nevertheless, the *hp* mutant shows a strong response to 539 nm light, presumably due to the relatively high φ-value (0.68) attained. That the *hp* mutant, in contrast to wild type, can accumulate high anthocyanin levels under these conditions again indicates a strong amplification of the LFR component. However, under 704 nm light, comparison of the slopes of the HIR component (above 1 μmol m^{-2} s^{-1}) of wild type and *hp* mutant suggest that the HIR is also amplified in the *hp* mutant. Under 729 nm light the low φ-value maintained leads to a low anthocyanin level at higher fluence rates and the absence of a HIR component, which is in agreement with results of Mancinelli (1990) for tomato seedlings. The indication of a dip in the fluence rate-response relationships at intermediate fluence rates of 539 and 649 nm light (wild type) and at high fluence rates of 729 nm light (wild type and *hp* mutant) (Fig. 4.5) is consistent with the predicted integrated Pfr level maintained by the lower fluence rate being higher than at higher fluence rates, due to protection of the PI pool from destruction (Fukshansky and Schäfer, 1983).

4.3.3. Hypocotyl growth

The study of hypocotyl growth inhibition indicates qualitively similar results to those discussed for anthocyanin synthesis, but comparatively it is more sensitive to light (Fig. 4.6). This is especially evident when comparing the 649 and 729 nm fluence rate-response relationships. In contrast to anthocyanin synthesis, the φ value of 0.05 maintained under 729 nm is sufficient to saturate inhibition of hypocotyl growth. Moreover, for hypocotyl growth, but not for anthocyanin synthesis, a high proportion of the maximum response is already reached at the lowest fluence rates (about 0.01 μmol m^{-2} s^{-1}) used. In contrast to anthocyanin synthesis, the *au* mutant also exhibits inhibition of hypocotyl growth by B. This agrees with the results of Adamse *et al.* (1988b) and again demonstrates the higher sensitivity of hypocotyl growth to light when compared to anthocyanin synthesis. The response of the *au* mutant to 693 nm light is not

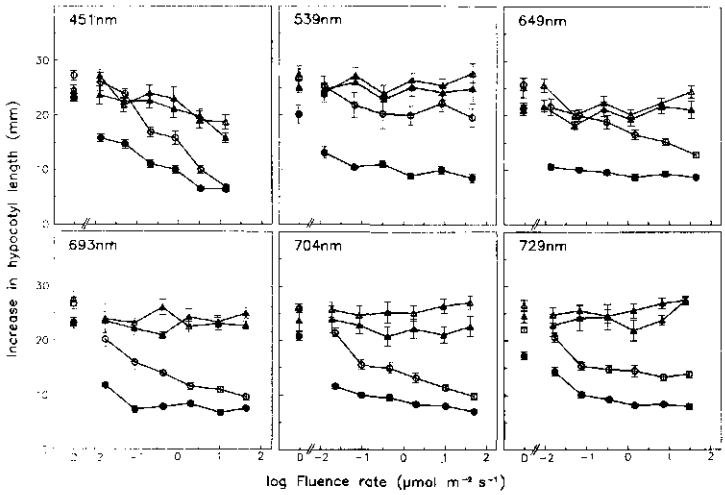


Figure 4.6. Fluence rate-response relationships for hypocotyl growth ± SE (2 replicates, 3 experiments) during a 24 h irradiation with 451 nm, 539 nm, 649 nm, 693 nm, 704 nm and 729 nm light for wild-type (○), *au*- (△), *hp*- (●) and *au,hp*- (▲) mutant tomato seedlings. D = darkness.

in agreement with the stimulation of the hypocotyl growth at fluence rates in the range 0.1-10 μmol m^{-2} s^{-1} reported by Lercari *et al.* (1990) for an *au* mutant in a different genetic background. The fact that D-grown *hp*-mutant seedlings are always significantly shorter than their corresponding wild-type seedlings may result from: inhibition caused by the green 'safelight' used during the first length measurement; a lower food reserve present in the somewhat smaller *hp*-mutant seeds; a greater response due to residual Pfr in the seeds.

4.3.4. Conclusion

The *hp* mutation is pleiotropic for phytochrome-controlled responses and is therefore not simply an anthocyanin response mutant. Although the *hp* mutant exhibits exaggarated phytochrome responses, the experiments discussed reveal that the characteristics of the phytochrome system controlling anthocyanin biosynthesis are similar in the *hp* mutant and wild type. The exaggerated response of the *hp* mutant compared to wild type fits the definition of 'responsiveness amplification' proposed by Mohr (1986) to describe the amplification of a phytochrome response as a result of preirradiation which excites either the B photoreceptor or phytochrome. We propose that the *hp* mutation is associated with an amplification step in the phytochrome transduction chain which, because the *au,hp* double mutant has severely reduced or no anthocyanin synthesis, reflects activity of the bulk PI pool. The fact that overexpressors of the *phyA* gene, of which PI is almost certainly the product, exhibit exaggerated phytochrome responses similar to the *hp* mutant

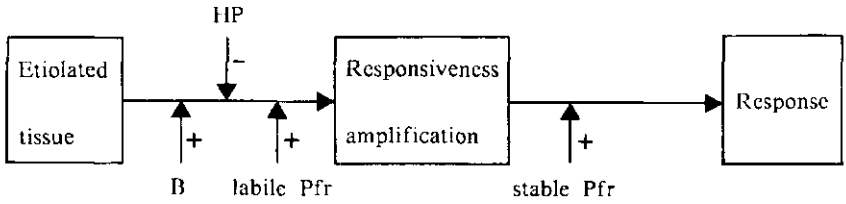


Figure 4.7. To accumulate anthocyanin a tomato seedling requires a certain 'degree of de-etiolation' (competence) which can be promoted (+) by the action of PI or the B photoreceptor (probably in coaction) and inhibited (–) by the product of the *Hp* gene (HP). Since the coaction between these components remains unclear, they are for simplicity drawn as acting independently. Once the state of responsiveness (degree of amplification) is set, the response is modulated by a stable phytochrome pool.

(Boylan and Quail, 1989), supports the argument in favour of involvement of the bulk PI pool. Combining this information with the finding that the phytochrome pool regulating the anthocyanin synthesis must be relatively stable (Fig. 4.3), the capacity for anthocyanin synthesis could be established by the bulk PI pool, while the anthocyanin synthesis is actually photoregulated *via* a stable phytochrome pool.

For normal development of the tomato seedling during de-etiolation coaction of the B photoreceptor and phytochrome is necessary (Chapter 3; Oelmüller and Kendrick, 1991). However, the *hp* mutant exhibits the maximum anthocyanin synthesis and hypocotyl growth inhibition in R alone (Chapter 3; Figs. 4.5 and 4.6). This recessive (loss-of-function) mutation therefore mimics the action of B. It is proposed that the phytochrome action in D-grown seedlings is under the constraint of an inhibitor: the product of the *Hp* gene (HP) and that B plays a role in overcoming this inhibiting effect (Fig. 4.7).

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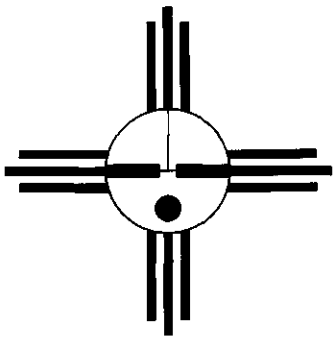


CHAPTER 5

THE RESPONSE OF PHOTOMORPHOGENETIC TOMATO MUTANTS TO END-OF-DAY FAR-RED LIGHT

Abstract. To study the response of light-grown plants to end-of-day (EOD) far-red light (FR) the following photomorphogenetic mutants were used: *aurea (au)*; high pigment *(hp)*; double mutant *(au,hp)*; the potential photomorphogenetic mutant *procera (pro)* and the isogenic wild type of tomato (*Lycopericon esculentum* Mill.) cv. Ailsa Craig. All of the genotypes tested exhibited a strong EOD FR response resulting in an increase in plant height, fresh weight of internodes and in the case of wild-type and *hp*-mutant plants a reduction in anthocyanin accumulation. However, it had no effect on their leaf area and chlorophyll content. Interposing a variable dark (D) period at the end of the white-light photoperiod before exposure to FR demonstrated that 50% of the EOD FR response on elongation growth and anthocyanin content was obtained for a D period of 4 h. This indicates that the phytochrome species regulating anthocyanin synthesis in the wild type and *hp* mutant and growth in wild type, *au*, *hp*, and *au,hp* has the same characteristics, being stable in the active FR-absorbing form. The EOD FR response on anthocyanin synthesis, determined by measurement of the anthocyanin content of young leaves at the same physiological stage of development demonstrated that the level of anthocyanin reached a minimum after 5 d EOD FR treatment. The potential of photomorphogenetic mutants, either selected by conventional means or genetically modified, for the control of plant growth is discussed.

5.1. INTRODUCTION

The control of plant development by light, photomorphogenesis, involves at least three different groups of photoreceptors, including phytochrome, a blue light (B)/UV-A photoreceptor (cryptochrome) and a UV-B photoreceptor (Mohr, 1986). Smith (1986) has proposed that one specific function of phytochrome in light-grown plants is related to the perception of an increased proportion of far-red light (FR) occurring in canopy shade light. This results in promotion of stem elongation in order to avoid shade light. One of the methods often used to trigger the phytochrome-mediated shade-advoidance process is to briefly irradiate plants with end-of-day (EOD) FR (Downs *et al.*, 1957).

Evidence has accumulated that multiple types of phytochrome exist: the light-labile (PI) and light-stable (PII) phytochrome types (Furuya, 1989; Tomizawa *et al.*, 1990). The assignment of specific functions to the distinct photoreceptors is being studied with the aid of photomorphogenetic mutants in which certain parts of the morphogenetic pathway are eliminated or altered (Koornneef and Kendrick, 1986; Adamse *et al.*, 1988c). Photomorphogenetic mutants have been isolated for a number of species, but the best characterized is the *aurea (au)* mutant of tomato (Adamse *et al.*, 1988b, c). Compared to its isogenic wild type it has no spectrophotometrically and immunochemically detectable phytochrome (<5%) in dark (D)-grown tissues (Koornneef *et al.*, 1985; Parks *et al.*, 1987) and exhibits reduced photoregulation of seed germination, anthocyanin synthesis, hypocotyl elongation and chlorophyll synthesis. At the molecular level it has greatly reduced phytochrome control of the chlorophyll *a/b*-binding protein gene *(cab)* expression (Sharrock *et al.*, 1988; Oelmüller *et al.*, 1989). In light-grown *au*-mutant plants about 60% of the wild-type level of phytochrome has been reported on the basis of dual-wavelength spectrophotometry (Adamse *et al.*, 1988b; López-Juez *et al.*, 1990b). Both wild-type and the *au*-mutant adult light-grown plants exhibit a quantitatively similar elongation growth response to EOD FR treatment indicating the presence of functional phytochrome in light-grown *au*-mutant plants (Adamse *et al.*, 1988b; López-Juez *et al.*, 1990b and Table 5.1). Although the most plausible inference is to ascribe this response to PII, which is predicted to accumulate in the mutant, the molecular nature of phytochrome detected in light-grown tissues (Adamse *et al.*, 1988b; López-Juez *et al.*, 1990b) has not yet been positively identified, since results using PI and PII specific antibodies are not yet available.

The monogenic recessive high pigment *(hp)* mutant shows characteristics opposite to the *au* phenotype: seedlings have an increased anthocyanin content (Chapters 2 and 3) and a reduced hypocotyl length in red light (R), B, UV-A (Chapter 3) and yellow light (Mochizuki and Kamimura, 1985) compared to wild type. Moreover, in light-grown plants the chlorophyll content is particularly high in immature fruit tissues (Sanders *et al.*, 1975) and mature fruits have a higher lycopene and carotenoid content than wild type (Kerr, 1965). Unlike wild type, the *hp* mutant does not require the activation of the B photoreceptor to exhibit high levels of anthocyanin synthesis and enables complete de-etiolation under R (Chapter 3). Since etiolated *au*-mutant and *au,hp* double-mutant seedlings, both deficient in PI, show no or a small (3% compared to *hp*) R/FR reversible anthocyanin response after a B pretreatment, respectively (Chapter 2), it was concluded that the PI pool regulates anthocyanin synthesis at the seedling stage and that the *hp* mutation does not result in the constitutive expression of genes involved in anthocyanin synthesis. The nature of the processes influenced by the *hp* mutation is unknown. The phytochrome content of comparable samples of etiolated *hp*-mutant and wild-type seedlings is similar (Chapter 3). Therefore the difference observed can not be explained by a higher absolute level of the active FR-absorbing form of phytochrome (Pfr). While initially it was proposed that the *hp* mutation

increases the sensitivity to Pfr (Chapter 2 and 3), subsequent research favours the modification of phytochrome amplification (Chapter 4).

This study investigates the influence of EOD FR treatment on the growth and pigmentation of the newly formed internodes and leaves of five tomato genotypes: (i) a *hp* mutant which has the same phytochrome content and a very high level of anthocyanin compared to wild type in seedlings; (ii) an *au* mutant deficient in the light-labile type of phytochrome (Koornneef *et al.*, 1985); (iii) the *au,hp* double mutant; (iv) a *pro* mutant having a phenotype similar to wild type treated with gibberellic acid (GA) (Jones, 1987; Jupe *et al,*. 1988) and (v) the isogenic wild type.

5.2. MATERIALS AND METHODS

5.2.1. Plant material

Seeds of the mutants and near isogenic wild type of tomato (*Lycopersicon esculentum* Mill.) cv. Ailsa Craig were harvested from plants grown in a greenhouse at the Department of Genetics, Wageningen, The Netherlands.

Seeds were then sown in sand with underlying compost, in earthenware seed pans. In experiments with the *au* and *au,hp* mutants all genotypes were pretreated on filter paper moistened with 25 μM gibberellin A_4 and A_7 (ICI, Yalding, UK) for 24 h at 25 ± 1°C. Seedlings were initially grown under a daily photoperiod of 14 h white light and 10 h D, at 20 ± 1°C and 70% relative humidity. After 8 d, uniform seedlings were selected and potted in 10 cm diameter plastic pots filled with potting compost. After a further 3 to 7 d seedlings were again selected, resulting in about 60% of the seedlings being used and the seedlings were transferred to a growth room at 23 ± 1 or 25 ± 1°C maintaining the same light regime. After a further 20 to 28 d EOD FR treatment was begun and repeated for up to 20 consecutive days. This consisted of transferring plants to a cabinet situated in the same growth room for a 20 min FR (+FR) irradiation at the end of the 14 h white light period, before replacing the plants on the staging. A dim green safelight was used when manipulating the plant material. The control plants received no EOD FR (-FR).

5.2.2. Growth measurement

At 0, 7 or 20 d from the start of EOD FR treatment the length of every internode was measured with a ruler. Total leaf area and fresh weight (fr wt) of the stems and leaves were determined. Leaf area was estimated with a leaf surface meter as described by Pieters (1984). The mature internode lengths of plants of the different genotypes growing in a greenhouse were measured with a ruler.

5.2.3. Anthocyanin assay

Samples of the youngest leaf were harvested, weighted and extracted with 1.2 ml acidified (1% HCl, wt/vol) methanol for 48 h in D with shaking. A Folch

partitioning (Folch *et al.*, 1957) was performed after adding 0.9 ml H_2O and 2.4 ml chloroform to the extracts and centrifugation for 30 min at 4800 rpm. The absorbance of the top phase was determined with a Beckman DU-64 spectrophotometer at 535 nm (A535). The results are expressed on a fresh-weight basis.

5.2.4. Chlorophyll assay

Chlorophyll contents were determined spectrophotometrically in 80% (vol/vol) acetone/water extracts, according to Bruinsma (1963). Comparably developed leaves were cut into small pieces and chlorophyll extracted. Further details are described in López-Juez *et al.* (1990a).

5.2.5. Light sources

White light was obtained from Philips TL 135/33 fluorescent tubes. The irradiance was 25 W m^{-2}; photosynthetically active radiation (400-700 nm) being 120 μmol m^{-2} s^{-1}. The emission spectrum of the tubes was determined with a spectral analyzer (Rofin-Sinar Laser UK Ltd., Weybridge, UK), controlled by a microcomputer system. The phytochrome photoequilibrium, (φ = Pfr/Pr+Pfr, where Pr is the R-absorbing form of phytochrome), is the proportion of total phytochrome maintained as Pfr at equilibrium by the light sources. It was calculated on the basis of the extinction coefficients and quantum yield data reported by Lagarias *et al.* (1987) and the formulae given by Mancinelli (1986). The calculated value established by the white light source was 0.82. Broad-band FR was produced by filtering the light from tungsten filament lamps through one layer of blue (No. 627) and one layer of red (No. 501) 3 mm plexiglas (Röhm and Haas, Darmstadt, Germany). The irradiance was 14 μmol m^{-2} s^{-1} in the range 700-780 nm and the 20 min exposure was sufficient to establish a value of φ = 0.05. Irradiance was measured with a photodiode meter, Optometer, type 80X (United Detector Technology Inc., Santa Maria, CA, USA).

5.2.6. Presentation of results

All experiments have been repeated with qualitatively similar results. The results presented are the mean ± standard error (SE) for individual or pooled experiments.

5.3. RESULTS

Table 5.1 shows that the *hp* mutant and wild type had similar chlorophyll contents, while the *au* and the *au,hp* mutants had about half of the chlorophyll content and were yellower in appearance. The chlorophyll *a*/*b* ratio was 5.0, 4.5, 3.0 and 3.1 for *au*, *au,hp*, *hp*, and wild type, respectively. Despite the difference in anthocyanin observed between wild type and the *hp* mutant at the seedling stage (Chapters 2, 3 and 4), they contained similar levels at the end of

Table 5.1. Comparison of the effect of a 20 min far-red light (+FR) pulse at the end of 7 daily photoperiods of 14 h white light (25 W m^{-2}) at 25°C with control plants (-FR) on chlorophyll and anthocyanin content of comparable leaf samples and increase (Δ) in plant height of wild-type (WT), *au*, *hp* and *au,hp* tomato plants (cv. Ailsa Craig).

Parameter		Genotype			
		WT	*hp*	*au*	*au,hp*
Δ height	+FR	146.3 ± 4.6	90.0 ± 2.0	200.7 ± 4.5	160.7 ± 5.1
(mm)	-FR	76.3 ± 2.4	39.0 ± 2.6	103.6 ± 2.5	82.0 ± 4.9
Chlorophyll	+FR	3.70 ± 0.38	3.40 ± 0.21	1.32 ± 0.03	1.85 ± 0.02
(mg/g fr wt)	-FR	3.12 ± 0.18	3.02 ± 0.22	1.62 ± 0.12	1.79 ± 0.16
Anthocyanin	+FR	0.57 ± 0.05	0.43 ± 0.04	ND*	ND*
(A535/g fr wt)	-FR	1.79 ± 0.15	1.67 ± 0.13	ND*	ND*

*ND = not detectable

the present experiments (Table 5.1). Clearly the *au,hp* double mutant is more like the *au* than the *hp* phenotype with respect to both chlorophyll and anthocyanin content. Therefore there is a clear correlation between these characteristics and the *au* mutation. However, despite these differences, all the genotypes responded to EOD FR with an increase in elongation growth (Table 5.1). In both the *hp* mutant and wild type there was a dramatic effect on the anthocyanin content of comparably developed leaves (Table 5.1). The time course of loss of anthocyanin content after EOD FR treatments (Fig. 5.1) probably represents the kinetics of anthocyanin dilution due to growth after an inhibition of biosynthesis. Although smaller in magnitude, a reduction in

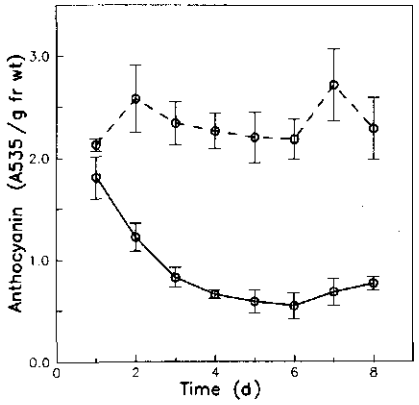


Figure 5.1. Anthocyanin content of comparably developed young leaves of wild-type tomato plants (cv. Ailsa Craig) grown under a daily photoperiod of 14 h white fluorescent light (25 W m^{-2}) at 25°C with (o——o) or without (o---o) 20 min end-of-day far-red light treatment.

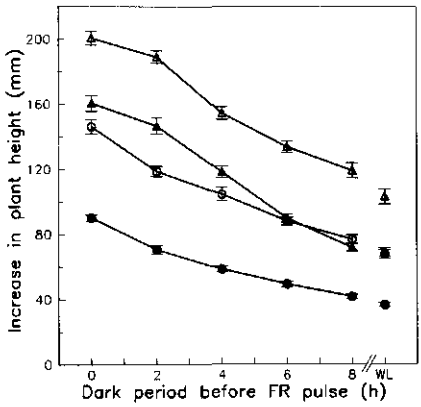


Figure 5.2. The effect of a 20 min far-red light (FR) pulse at different times after the end of 7 daily photoperiods of 14 h white fluorescent light (25 W m^{-2}) at 25°C on the increase in plant height of tomato wild-type (○), *au* (△), *hp* (●) and *au,hp* (▲) tomato plants (cv. Ailsa Craig). Control without FR (WL).

anthocyanin was also observed in older leaves (data not shown).

The characteristics of phytochrome regulation of the EOD FR response were further studied by interposing a D period at the end of the day before exposure to FR. A delay of 4 h still results in approximately 50% of the EOD FR response on elongation growth (Fig. 5.2) for *au*, *au,hp*, *hp* and wild type and anthocyanin content for *hp* and wild type (Fig. 5.3). This means that Pfr,

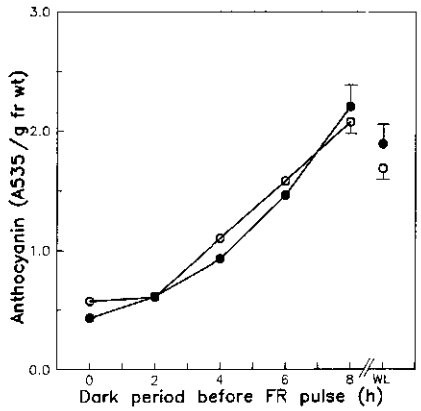


Figure 5.3. The effect of a 20 min far-red light (FR) pulse at different times after the end of 7 daily photoperiods of 14 h white fluorescent light (25 W m^{-2}) at 25°C on the anthocyanin content of tomato wild-type (○) and *hp* (●) tomato plants (cv. Ailsa Craig). Control without FR (WL).

Table 5.2. Comparison of the effect of a 20 min far-red light (+FR) pulse at the end of 20 daily photoperiods of 14 h white light (25 W m^{-2}) at 23°C with control plants (–FR) on the increase (Δ) in plant height, stem growth rate, Δ stem fresh weight and leaf area of wild-type (WT), *hp* and *pro* tomato plants (cv. Ailsa Craig).

Parameter		Genotype		
		WT	*hp*	*pro*
Δ height	+FR	123.4 ± 6.0	96.3 ± 5.6	421.7 ± 15.6
(mm)	–FR	80.9 ± 4.0	73.9 ± 5.6	285.3 ± 9.1
Stem growth	+FR	6.0 ± 0.3	4.3 ± 0.3	21.9 ± 0.8
rate (mm/d)	–FR	4.0 ± 0.2	3.7 ± 0.3	14.3 ± 0.5
Δ stem fr wt	+FR	5.95 ± 0.73	4.03 ± 0.33	14.91 ± 2.00
(g)	–FR	4.60 ± 0.56	3.32 ± 0.28	11.90 ± 1.59
Leaf area	+FR	481.3 ± 41.1	523.3 ± 60.2	645.2 ± 61.6
(cm^2)	–FR	464.8 ± 38.9	507.7 ± 58.3	675.9 ± 66.2
Leaf fr wt	+FR	16.30 ± 1.89	14.35 ± 1.64	18.58 ± 1.94
(g)	–FR	15.00 ± 1.66	13.14 ± 1.49	17.77 ± 1.81

involved in this response must still be present at that time, *i.e.* the pool of phytochrome regulating the EOD FR response is relatively stable in the Pfr form.

In a subsequent series of experiments we also studied the influence of EOD FR, not only on the *hp* mutant and wild type, but also on the *pro* mutant. In addition to an increase in internode length during the experimental period, increase in stem fresh weight, leaf area and leaf fresh weight were determined (Table 5.2). The *pro* mutant exhibited a normal EOD FR response on elongation growth. There was no influence of EOD FR on leaf area and leaf fresh weight of the three genotypes studied (Table 5.2).

The mean internode lengths of plants growing in the greenhouse during the summer was also determined (Table 5.3). The *hp* mutant had the shortest

Table 5.3. The mean internode length of mature wild-type (WT), *hp*-, *au,hp*-, *au*- and *pro*-mutant tomato plants (cv. Ailsa Craig) growing in a greenhouse during the summer.

Genotype	Internode length (cm)
WT	5.30 ± 0.27
hp	5.23 ± 0.35
au,hp	5.85 ± 0.28
au	6.96 ± 0.29
pro	7.38 ± 0.47

internode length, but it was not significantly different from that of wild type. The *pro* mutant had the longest internode length followed by the *au* mutant and the *au,hp* double mutant, which had slightly longer internodes than wild type.

The phenotype of the *pro* mutant is remarkably similar to that of the wild type treated with GA (Jones, 1987; Jupe *et al.*, 1988). However, this mutant does not have increased GA levels (Jones, 1987). It was suggested by Adamse *et al.* (1988c) that the *pro* mutant could be similar to the cucumber *lh* mutant and is a possible candidate for a mutant deficient in the function of the light-stable type of phytochrome. However, in contradiction to this possibility, *pro* exhibits an EOD FR elongation response (Table 5.2), not shown by the *lh* mutant cucumber (López-Juez *et al.*, 1990a).

If the proposal of García-Martínez *et al.* (1987) that GAs are involved in the EOD FR response is correct, the *pro* mutant can not be saturated for the particular GA response involved, since it exhibits a typical EOD FR response (Table 5.2).

5.4. DISCUSSION

Despite the inefficient de-etiolation process of the *au* mutant (Peters *et al.*, 1991b), light-grown plants exhibit an apparently normal EOD FR response in terms of elongation growth (Table 5.1). However, the *au* mutant has yellow leaves, lacks detectable anthocyanin (Table 5.1) and has slightly longer internodes than wild type under greenhouse conditions (Table 5.3). This suggests that the phytochrome regulating these photomorphogenic characteristics is deficient in light-grown plants. These observations lend support to the hypothesis that chlorophyll biosynthesis and the EOD FR responses are under the control of different phytochrome pools. These pools, which are thought to be light-labile and light-stable may be PI and PII, respectively. Since the phytochrome regulating the EOD FR is apparently stable in the Pfr form, it is tempting to speculate that the EOD FR response is regulated by PII.

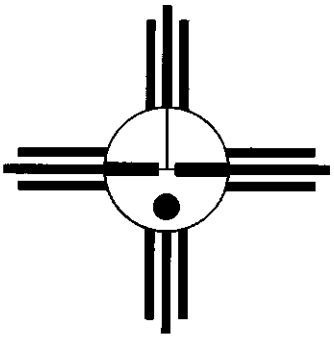
During normal development of tomato, de-etiolation is achieved, not only as a result of photoactivation of phytochrome, but also by its coaction with the B photoreceptor. It appears that activation of the B photoreceptor results in an amplification of the phytochrome response. In fact, it is the coaction of the B photoreceptor and phytochrome which enables the *au* mutant to become sufficiently de-etiolated so that it can survive (Peters *et al.*, 1991b; Oelmüller and Kendrick, 1991). However, the *au* mutation has a strong residual effect as indicated by its reduced chlorophyll levels and its inability to form anthocyanin in light-grown plants. Taken together, these observations give credence to the notion of discrete functional phytochrome pools in plants. While PII may be a type of phytochrome which functions predominantly in light-grown plants, PI is

essential for plastid development during de-etiolation and it can be envisaged that this role is maintained even in light-grown plants.

The *hp* mutant is in many ways opposite to the *au* mutant and resembles transgenic tomato plants which overexpress the oat PI gene (Boylan and Quail, 1989). However, the phytochrome content of the *hp* mutant is the same as wild type and studies at the de-etiolation stage have led to the conclusion that the *hp* mutation is associated with an amplification step in the phytochrome transduction chain (Chapter 4). Plants with *hp* characteristics at the seedling stage (*i.e.* short hypocotyls with high anthocyanin levels) were obtained when high levels of an oat PI gene was expressed in tomato (Boylan and Quail, 1989). Light-grown transgenic plants classified as either null or as low-level expressors resembled wild type, while high-level expressors, in contrast to *hp*, were extreme dwarfs, with dark-green foliage and fruits. Regardless of whether the height of the adult plant was normal or dwarf, seedlings expressing oat PI all had short hypocotyls at the seedling stage. The overproduction of oat PI results in the persistence of oat PI in light-grown plants, demonstrating that PI can be biologically active in fully green tissue. This does not necessarily mean that tomato PI plays a dominant role in elongation growth of light-grown wild type plants, since quantitative differences in the rate and the extent of degradation of the Pfr forms of tomato and oat PI in the transgenic plants were observed. Boylan and Quail point out that the *au* mutant, wild type and transgenic PI overexpressors represent a continuum of phenotypic expression in response to increasing levels of PI.

What possibilities do these studies of photomorphogenetic mutants and transgenic plants provide for the regulation of plant growth and development in horticulture? With an increase in the use of supplementary lighting in greenhouses (Vince-Prue and Canham, 1983) and the use of plastic mulches (Decoteau *et al.*, 1988) dramatic deviations from the spectral quality of daylight can occur which can be perceived by phytochrome or the B photoreceptor. Most recessive mutations are loss-of-function mutants. The observations that de-etiolated *(det)* mutants of *Arabidopsis* initiate de-etiolation in D (Chory *et al.*, 1989b) and that the *hp* mutant no longer requires B for normal development (Chapter 3), suggest that there is a negative control of growth. In other words, the photoreceptors can be envisaged as functioning in the removal of some inhibitory substance. One possible scenario is that the mutations result in a reduced rate of synthesis of this hypothetical inhibitor. It has been known for a long time that the spectral quality of light used for supplementary irradiation can result in abnormal growth and development of many species. However, the potential variation seen here for photomorphogenetic mutants suggests that using conventional breeding techniques, or genetic modification, it should be possible to produce plants which exhibit 'normal' growth under light sources such as high-pressure sodium, which are deficient in B compared to normal daylight.

Acknowledgements. We thank Corrie J. Hanhart and Sebastiaan Verduin for valuable technical assistance; the phytotron and greenhouse staff for cultivation of the plants.



CHAPTER 6

PHYTOCHROME CONTENT AND HYPOCOTYL GROWTH OF LONG-HYPOCOTYL MUTANT AND WILD-TYPE CUCUMBER SEEDLINGS DURING DE-ETIOLATION

Abstract. Photoinhibition of hypocotyl growth has been studied in a cucumber (*Cucumis sativus* L.) long-hypocotyl *(lh)* mutant and its near isogenic wild type. Hypocotyl-growth rate of the mutant and wild type was the same in darkness (D) and was equally inhibited by continuous far-red light (FR). However, under continuous red light (R) the mutant was dramatically less inhibited, resulting after 120 h R in hypocotyls being almost twice as long as those of wild type. Although smaller in absolute length, similar results were obtained under continuous blue light (B). *In vivo* spectrophotometric phytochrome measurements with Norflurazon-treated plants showed that D-grown *lh*-mutant and wild-type hypocotyl hooks contain similar amounts of phytochrome, while after 4 d FR, R and B, significantly less phytochrome was detected in the mutant than in the wild type. After 4 d in D, morphologically similar *lh*-mutant and wild-type plants were exposed to R, resulting in the depletion of the light-labile phytochrome pool (PI) by destruction. During the first 2.5 h the half life for depletion of PI for mutant and wild type was similar (about 30 min) at 25°C. However, after 3 h R a significantly lower phytochrome steady-state level was reached in the mutant than the wild type. When plants were pretreated for 4 d with FR before exposure to R, a similar difference in phytochrome steady-state level was observed. Phytochrome resynthesis was measured in seedlings after pretreatment with R and B. Although the rate of resynthesis was slower after B, there were no significant differences in its rate between mutant and wild type. Despite PI destruction and resynthesis being identical in wild type and mutant, the phytochrome level reached after de-etiolation (the PI steady-state plus the light-stable phytochrome [PII]) is different. We propose that the *lh* mutant exhibits reduced phytochrome function in light-grown plants due to depletion of a stable phytochrome pool.

6.1. INTRODUCTION

Plants utilize light both as an energy source for photosynthesis and as a stimulus to trigger a large number of developmental processes (Kendrick and Kronenberg, 1986; Furuya, 1987). This latter light-dependent development of plants is termed photomorphogenesis and several photoreceptors are involved, including the red light (R)/far-red light (FR) absorbing phytochrome, the blue

light (B)/UV-A absorbing cryptochrome and a UV-B absorbing pigment (Mohr, 1986). The best characterized photoreceptor is phytochrome, which regulates developmental events ranging from germination to flowering. It is a chromoprotein, consisting of an apoprotein with a linear tetrapyrrole attached to the N-terminal domain (Vierstra and Quail, 1986), which exists as a dimer in solution (Tokutomi *et al.*, 1989). At least two physiologically and spectrophotometrically distinguisable phytochrome types exist: a light-labile phytochrome type (PI) which is abundant in etiolated tissue and undergoes destruction upon illumination and a light-stable phytochrome type (PII) (for review see Furuya, 1989). Since the absorption spectra of PI and PII are very similar (Tokuhisa *et al.*, 1985; Abe *et al.*, 1985) it is difficult to distinguish the different roles of these photoreceptors in the photocontrol of plant development (Furuya, 1989). The assignment of specific functions to the distinct phytochrome types is therefore being studied with the aid of photomorphogenetic mutants modified with respect to either PI or PII (Adamse *et al.*, 1988c) or transgenic plants overexpressing the PI gene (Boylan and Quail, 1989, Kay *et al.*,1989; Keller *et al.*, 1989). Photomorphogenetic mutants have been isolated for several plant species (Adamse *et al.*, 1988c; Tomizawa *et al.*, 1990): the *aurea (au)*, the high pigment *(hp)* and the *au,hp* double mutant of tomato (Adamse *et al.*, 1988c; Peters *et al.*,1991b); the long-hypocotyl *(hy1, hy2, hy3, hy4, hy5, hy6)* mutants (Koornneef *et al.*, 1980; Chory *et al.*, 1989a) and de-etiolated *(det)* mutants which initiate de-etiolation in darkness (D) (Chory *et al.*, 1989b) of *Arabidopsis*; the long-stemmed *lv* mutant of pea (Reid and Ross, 1988; Nagatani *et al.*, 1990).

In this study we used the long-hypocotyl *(lh)* mutant of cucumber, which was originally isolated on the basis of its elongated hypocotyl in white light (Koornneef and van der Knaap, 1983). The *lh* mutant and the wild type have a similar rate and percentage of seed germination, hypocotyl growth rate in D, R/FR reversibility of hypocotyl growth in etiolated seedlings, phytochrome content in seeds and phytochrome synthesis in D which precedes germination (Adamse *et al.*, 1987). Moreover, spectrophotometric measurements of phytochrome *in vivo* (Adamse, 1988a) and *in vitro* (Nagatani *et al.*, 1989), along with their immunochemical reactivity against the monoclonal antibody mAP5 (Nagatani *et al.*, 1989) indicate that the *lh* mutant and wild type contain a similar amount of phytochrome in etiolated tissue. De-etiolated seedlings of the *lh* mutant, compared to wild type fail to respond to R, show no R/FR reversibility of hypocotyl growth (Adamse *et al.*, 1987), lack the end-of-day (EOD) FR response on stem elongation (Adamse *et al.*, 1988a; López-Juez *et al.*, 1990a) and contain 35-40% of the spectrophotometrically detectable phytochrome in wild-type flower petals and Norflurazon-bleached leaves (Adamse *et al.*, 1988a). Based on these findings Adamse *et al.* (1988a) proposed that de-etiolated seedlings of the *lh* mutant lack the function attributable to a light-stable phytochrome type due to deficiency of the photoreceptor, which accumulates in light-grown seedlings. However, this hypothesis was not confirmed by immunochemical and biochemical investigations by Nagatani *et al.* (1989). These workers demonstrated that young de-etiolated seedlings

contain similar amounts of phytochrome in mutant and wild-type extracts. Since in both genotypes about one quarter of the phytochrome could be immunoprecipitated by an antibody which recognizes PI, they concluded that the wild type and the *lh* mutant had similar PI and PII levels, although there was no positive identification of PII. Nagatani *et al.* (1989) proposed two possible explanations for the *lh* phenotype: (i) the mutation changes the function of phytochrome without changing its stability and spectrophotometrical characteristics; (ii) the mutation results in modification of the signal transduction chain between the photoreceptor active in light-grown plants and physiological responses.

In this paper we report on experiments designed to determine the phytochrome content of hypocotyls during de-etiolation, the kinetics of phytochrome destruction and synthesis in hypocotyls, as well as hypocotyl growth kinetics under different light regimes of the *lh* mutant and its isogenic wild type.

6.2. MATERIALS AND METHODS

6.2.1. Plant material

Seeds of the *lh* mutant and near isogenic wild type of *Cucumis sativis* L. were used in the experiments. The original mutant was found in the progeny of X-ray irradiated *Cucumis* plants of complex hybrid origin (van der Knaap and de Ruiter, 1978). Koornneef and van der Knaap (1983) showed that this monogenic recessive mutant was allelic with a similar mutant described and named *lh* by Robinson and Shail (1981). The original mutant (van der Knaap and de Ruiter, 1978) was crossed with the cucumber cultivar Stereo (De Ruiter & Zonen, Bleiswijk, The Netherlands). Mutant *(lh,lh)* and wild type *(Lh,Lh)* lines derive from the same F_4 plant, thus a reasonable amount of isogenicity is present between them. The subsequent generations were obtained by selfing and seeds from the F_7, F_8 and F_9 generations were used in the present experiments.

Seeds were selected and imbibed in distilled water for 10 min. Sixteen seeds were sown per standard plastic box (interior dimensions 9.5x9.5x4.7 cm) containing 8 layers of unbleached tissue (Aldi, Germany) and 30 ml distilled water. The seeds were arranged equidistantly in 4 rows with the radicle poles pointing in the same direction. Growth temperature during the light treatments and in D was 25.0 ± 0.3°C. For phytochrome measurements seedlings were treated with 10^{-5} M Norflurazon (Sandoz AG, Basel, Switzerland).

6.2.2. Light sources

For continuous light treatments, standardized light fields were used: red light (R, λ_{max} at 658 nm, half bandwidth 24 nm, fluence rate 6.8 W m^{-2}, φ = 0.80), far-red light (FR, λ_{max} at 740 nm, half bandwidth 100 nm, fluence rate 3.5 W m^{-2}, φ = 0.03) and blue light (B, λ_{max} at 450 nm, half bandwidth 45 nm, fluence rate

10 W m^{-2}, φ = 0.38) (Mohr and Drumm-Herrel, 1981). Estimates of the phytochrome photoequilibrium at different wavelengths (φ = proportion of total phytochrome (Ptot) in the FR-absorbing form, Pfr/Ptot) are based on the *in vivo* absorption spectra for 124 Kd *Avena* phytochrome published by Vierstra and Quail (1983).

For the degradation kinetics of total phytochrome another R field was used (λ_{max} at 658 nm, half bandwith 24 nm, fluence rate 0.68 W m^{-2}, φ = 0.80).

6.2.3. Phytochrome determination

Phytochrome measurements were carried out using a custom-built dual-wavelength spectrophotometer (Gross *et al.*, 1984). Interference filters from Schott (Mainz, Germany) served to isolate measuring and actinic beams (DIL filters at 729 nm and 793 nm and AL filters at 661 nm and 757 nm, respectively). Twenty hypocotyl-hook sections (7 mm in length and about 200 mg fresh weight) were cut, packed into a pre-cooled aluminium cuvette with an internal diameter of 6 mm under dim-green safelight and stored at 0°C prior to measurement at 0°C. In each experiment 1 to 4 samples were measured and the experiments were repeated 3 to 9 times. The pooled results are expressed as mean total phytochrome (Ptot) ± standard error (SE).

6.2.4. Hypocotyl length measurements

The hypocotyl length was measured to the nearest 0.5 mm with a ruler. In each experiment 32 seedlings were measured and the experiments were repeated 2 to 5 times. The pooled results are expressed as mean hypocotyl length ± SE.

6.3. RESULTS

6.3.1. Hypocotyl growth

In continuous D no difference was observed in hypocotyl-growth rates between the *lh* mutant and its near isogenic wild type (Fig. 6.1). After 72 h FR, R or B the mutant hypocotyl length is slightly longer than wild type, due to the accumulative effect of reduced inhibition during 3 d irradiation prior to the first measurement and/or a slight effect of the irradiation on the rate of germination. In spite of this, the mutant and wild-type hypocotyl growth rates (Fig. 6.1) and the growth increments between 96 h and 120 h after sowing (Table 6.1) are the same under continuous FR. However, under continuous R, the *lh* mutant and wild type exhibit a similar growth rate during the first 24 h of measurement (from 72 to 84 h after sowing), but subsequently the mutant exhibits a dramatic reduction in inhibition, resulting in hypocotyls twice as long as those of wild type after 120 h R (Fig. 6.1). Although shorter in absolute length, similar results as in R were obtained in B (Fig. 6.1). In Table 6.1 the growth increment between 96 and 120 h after sowing is presented for seedlings grown in: (i) continuous D, R, FR and B; (ii) 96 h D before transfer to 24 h R, FR and B; (iii) 96 h R to 24 h B, 96 h FR to 24 h R and 96 h B to 24 h R. In

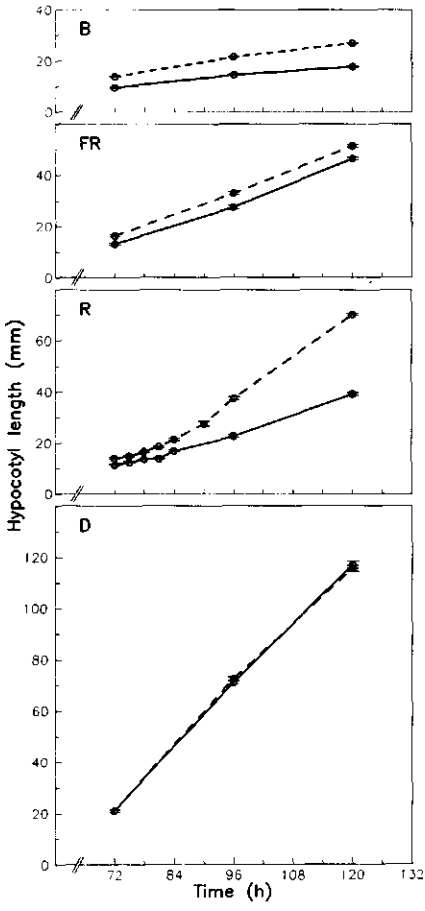


Figure 6.1. Hypocotyl elongation of *lh*-mutant (o---o) and wild-type (o——o) cucumber seedlings in continuous blue light (B), far-red light (FR), red light (R) and darkness (D) at 25°C. The data represent the mean ± SE.

addition to Fig. 6.1, Table 6.1 shows the same effect of FR on growth of wild type and *lh* mutant, while R and B result in a decreased growth inhibition of the mutant compared to wild type.

6.3.2. Phytochrome content

The *in vivo* phytochrome content of 4 d old D-, FR-, R- and B-grown hypocotyls treated with the herbicide Norflurazon was measured spectrophotometrically. Treatment with Norflurazon leads to chlorophyll depletion by photobleaching as a result of its inhibition of carotenoid biosynthesis (Reiss *et al.*, 1983). Dark-grown *lh*-mutant hypocotyl hooks appeared to contain slightly more

Table 6.1. The increase in hypocotyl length of *lh*-mutant and wild type cucumber seedlings after different light treatments at 25°C. The SE's were all < 5% of the mean. Darkness (D), red light (R), far-red light (FR) and blue light (B).

Light treatment from sowing	Growth increment between 96 and 120 h after sowing (mm)	
	Wild type	*lh* mutant
120 h D	45.8	43.1
120 h R	16.5	32.6
120 h FR	18.9	18.4
120 h B	3.1	5.3
96 h D → 24 h R	16.1	26.6
96 h D → 24 h FR	23.8	23.8
96 h D → 24 h B	4.9	9.1
96 h R → 24 h B	12.7	15.3
96 h FR → 24 h R	7.2	14.4
96 h B → 24 h R	6.0	11.4

phytochrome (questionably significant) compared to wild type, while after 4 d FR, R or B less phytochrome was detected in the mutant (Table 6.2) after all treatments. To gain more information about the nature of this apparent phytochrome deficiency in the light-grown *lh* mutant, experiments were carried out to determine rates of phytochrome destruction and resynthesis.

6.3.3. Phytochrome destruction

After 4 d in D the *lh*-mutant and wild-type seedlings were of the same height (Fig. 6.1), morphologically similar and contained comparable amounts of phytochrome (Table 6.2). Exposing these plants to R resulted in a depletion of the PI pool by destruction, which was similar for wild type and mutant (Fig. 6.2). However, after 3 h R a significantly lower phytochrome level was reached in the mutant. Pretreatment of the seedlings with 4 d FR before exposure to R led to qualitatively similar results (Fig. 6.2). The depletion of Ptot was

Table 6.2. Total phytochrome content (Ptot) ± SE of *lh*-mutant and wild type cucumber seedlings after 4 d continuous far-red light (FR), red light (R), blue light (B) and darkness (D) from the time after sowing at 25 °C.

Treatment	Ptot 10^{-3} Δ(ΔA)	
	Wild type	*lh* mutant
D	12.05 ± 0.35	13.10 ± 0.43
FR	3.20 ± 0.29	2.47 ± 0.18
R	0.69 ± 0.04	0.33 ± 0.03
B	0.53 ± 0.03	0.15 ± 0.01

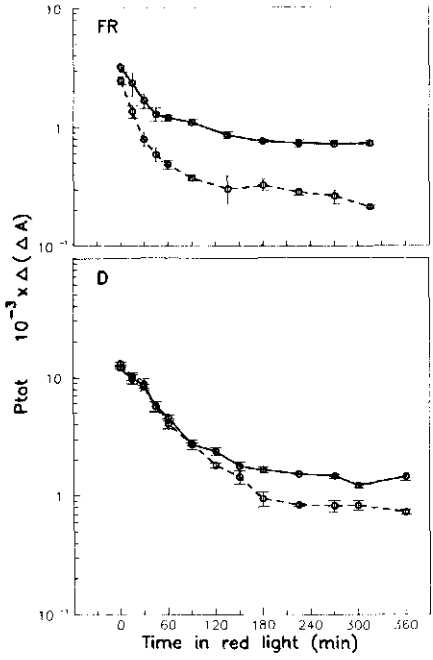


Figure 6.2. Destruction kinetics of total phytochrome (Ptot) ± SE in the hypocotyl-hook region of Norflurazon-treated *lh*-mutant (o---o) and wild-type (o——o) cucumber seedlings after the onset of red light (R). Before the onset of R, at time zero, the plants were kept from the time of sowing in far-red light (FR) or darkness (D) for 5 d at 25°C.

calculated by plotting Ptot at time (t) minus Ptot at the steady state (t = ∞) against time (according to the method of Schäfer *et al.*, 1972) and was a first order reaction (data not shown) with a half life of about 32 and 30 min after D pretreatment; 29 and 31 min after FR pretreatment for the wild type and the *lh* mutant, respectively at 25°C.

6.3.4. Phytochrome resynthesis

In addition to phytochrome destruction during irradiation another parameter which affects the steady-state pool size of PI is the rate of resynthesis. To determine the role played by resynthesis, plants were transferred to D after either 4 d R or B. The rate of resynthesis of PI after B was about 50% of that observed after R (Fig. 6.3). This slower rate of resynthesis following B explains the very low steady-state phytochrome level in the *lh* mutant after 4 d B as compared to R (Table 6.2). The slightly more rapid rate of resynthesis in the *lh* mutant than wild type could account for the slightly higher phytochrome level observed in D-grown seedlings (Table 6.2), but it is questionably significant.

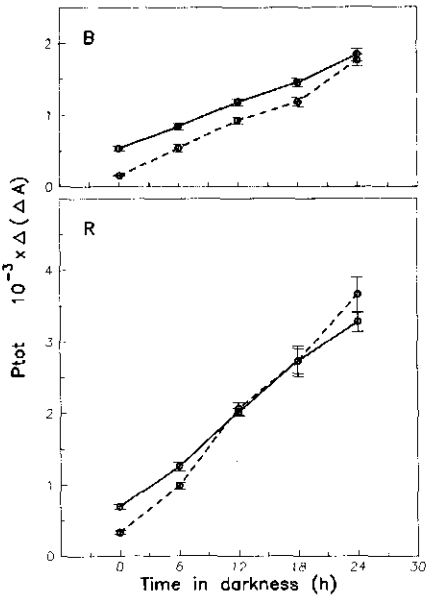


Figure 6.3. Resynthesis of total phytochrome (Ptot) ± SE in the hypocotyl-hook region of Norflurazon-treated *lh*-mutant (o---o) and wild-type (o——o) cucumber seedlings after transfer to darkness (D). Before transfer to D the plants were kept from the time of sowing in blue light (B) or red light (R) for 4 d at 25°C.

6.4. DISCUSSION

The hypocotyl-growth kinetics in Fig. 6.1 show that the inhibition of growth by R is maintained in the wild type, but gradually lost during de-etiolation in the *lh* mutant. This observation is consistent with the hypothesis that hypocotyl growth of cucumber seedlings is initially regulated by the bulk pool of phytochrome present in D-grown seedlings (PI), while during de-etiolation PII becomes increasingly important. It is interesting to note that there is little difference between the *lh* mutant and wild type in effectiveness of FR in inhibiting hypocotyl growth (Fig. 6.1 and Table 6.1). The high irradiance reaction (HIR) for inhibition of hypocotyl growth during de-etiolation has been attributed to the labile PI pool (for review see Cosgrove, 1986). These results indicate retention of the HIR in the FR region of the spectrum until at least 120 h after sowing in both the *lh* mutant and wild type (Fig. 6.1), a response presumably due to the residual PI pool. We envisage a gradual transition during de-etiolation in which the seedlings become increasingly responsive to PII. After de-etiolation, the wild-type hypocotyl responds in a way typical of

internodes and exhibits an elongation response to EOD FR, which is absent in the *lh* mutants (Adamse *et al.*, 1987, López-Juez *et al.*, 1990a).

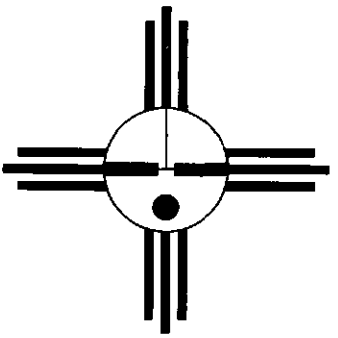
In this chapter it is demonstrated that the rates of phytochrome destruction and resynthesis of PI after destruction are similar in the *lh* mutant and wild type (Figs. 6.2 and 6.3). These observations make it unlikely that the lower steady-state level of phytochrome after 3 h R (Fig. 6.2) and after 4 d FR, R or B (Table 6.2) in the mutant can be attributed to the bulk PI pool. The fact that the size of the pool undergoing destruction in R after transfer from D or FR is quantitatively identical supports the conclusion that there is no difference between the *lh* mutant and wild type with respect to the PI pool. Furuya (1989) has demonstrated that the down-regulation of the PI gene in pea is regulated by a light-stable phytochrome pool. Here we show that the rate of phytochrome resynthesis is 50% lower after B irradiation which suggests that cryptochrome also plays a role in the regulation of the PI gene(s). Since it is well known that cryptochrome sensitizes seedlings to phytochrome action and if down regulation of the PI gene(s) is phytochrome regulated in cucumber, then this could explain the reduced rate of resynthesis. The steady-state phytochrome level attained under irradiation is composed of the PI steady state plus the PII pool (Furuya, 1989). Therefore, the difference in phytochrome level observed in the *lh* mutant could arise because it is deficient in PII. If this is the case, those phytochrome-dependent responses missing or severely depleted in the light-grown *lh* mutant plants could be attributed to PII.

While the phytochrome measurements presented here agree with those of Adamse *et al.* (1987) in flower petals and Norflurazon-bleached leaves, they are not supported by the study of Nagatani *et al.* (1989). These latter workers extracted phytochrome from the 24 h R de-etiolated *lh*-mutant and wild-type seedlings, which would be predicted to have different phytochrome levels on the basis of the present study and found no differences in its amount or immunological properties. However, the phytochrome extracted was not recognized by pea PII antibody (mAP11) and therefore no positive identification of cucumber PII was possible.

Clearly it is important that we are able to identify PI and PII in cucumber. To do this new antibodies are required and it is hoped that such antibodies will become available now the sequence of PII gene(s) is/are known (Sharrock and Quail, 1989). We conclude that the *lh* mutant of cucumber is still a possible contender for the first PII photoreceptor mutant to be identified[1].

Acknowledgements. We thank Dr. Maarten Koornneef and members of the Genetics Department, Wageningen Agricultural University, for provision of the seeds used in this study. This work was supported by a Travel Award to J.L.P. from the Netherlands Organization for the Advancement of Research (NWO) and from SFB 206 University of Freiburg, W. Germany.

[1]The conclusions drawn here have been recently substantiated with new antibodies and the findings are discussed in the following chapter.



CHAPTER 7

GENERAL DISCUSSION

In this chapter the results of Chapters 2 through 6 are integrated and reviewed in the light of relevant literature. Firstly, the concept that individual members of the phytochrome family have discrete physiological roles is discussed and an attempt made to assign physiological roles to them. Secondly, the possibility that the high-pigment *(hp)* mutant of tomato is a phytochrome transduction-chain mutant is outlined and its characteristics are compared with those of other potential transduction-chain mutants.

7.1. DO DIFFERENT PHYTOCHROMES HAVE DIFFERENT ROLES?

In order to answer the question whether different phytochromes have different physiological roles, phytochrome mutants altered in the abundance or functional integrity of particular phytochrome types have been studied. Detailed investigation of the phytochrome types present and their properties in such mutants allows the role(s) played by the phytochrome type lacking in a mutant to be assessed.

7.1.1. Characteristics of potential phytochrome-deficient mutants

In the preceding chapters two phytochrome mutants, the tomato *aurea (au)* and the cucumber long-hypocotyl *(lh)* mutants, have been characterized. The results presented in this thesis, together with data found in the literature are summarized in Table 7.1. The *au* mutant shows a reduction in: germination in darkness (D), hypocotyl growth in blue light (B), UV, and red light (R), anthocyanin synthesis, chlorophyll content and the photoregulation of the transcript levels of the genes encoding the chlorophyll *a/b*-binding protein *(cab)*, plastocyanin *(pet* E) and subunit II of photosytem I *(psa* D). The *lh* mutant shows normal D germination and inhibition of hypocotyl growth in B, UV and far-red light (FR) of etiolated plants, but deviates from the wild-type behaviour in the case of hypocotyl-growth inhibition in R in etiolated plants and

Table 7.1. Phenotypic characteristics of the tomato *au* and cucumber *lh* mutants when compared to their isogenic wild types.

Response	Tomato	Cucumber
	au	*lh*
Seed germination		
D	−[c,g,i]	+[r]
R/FR reversible promotion (LFR)	+[c,i] / −[g]	
FR inhibition (HIR)	−[c,i,g]	
Etiolated plants		
Inhibition hypocotyl growth in B and UV	−[a,g,h,m,o]	+[r,v]
Inhibition hypocotyl growth in R	−[a,g,m,o]	−[r,v]
Inhibition hypocotyl growth in FR	+[a,g] / −[o]	+[r,v]
Anthocyanin content	−[b,g,o]	
PAL activity	+[d]	
Chlorophyll content	+[e]	
mRNA: *cab*	−[k,p]	
psa D and *pet* E	−[k]	
rbcS	+[k,p]	
De-etiolated plants		
EOD FR response	+[a,j,n]	−[s,u]
R:FR photon ratio response	+[f,q]	−[q,t]
Anthocyanin content	−[f,j,m,n]	
Chlorophyll content	−[g,j]	+[r] / −[u]
Chloroplast development	−[g]	
Chlorophyll *a/b* ratio	−[g,j]	+[r]
mRNA: *cab*, *psa* D, *pet* E in WL	+[l]	
cab in B	+[l]	
cab in R	−[l]	
rbcS	−[p]	
Simulated phototropism	+[a]	−[r]

(+) response exhibited at wild-type level; (−) response different from wild type; when no data are available space is left open. LFR, low fluence response; HIR, high irradiance response; WL, white light; *rbcS*, gene coding for the small subunit of rubisco.
[a]Adamse *et al.* (1988b); [b]Adamse *et al.* (1989); [c]Georghiou and Kendrick (1991); [d]Goud *et al.* (1991); [e]Ken-Dror and Horwitz (1990); [f]Kerckhoffs *et al.* (1992); [g]Koornneef *et al.* (1985); [h]Lercari *et al.* (1990); [i]Lipucci di Paola *et al.* (1988); [j]López-Juez *et al.* (1990b); [k]Oelmüller *et al.* (1989); [l]Oelmüller and Kendrick (1991); [m]Peters *et al.* (1989); [n]Peters *et al.* (1992a); [o]Peters *et al.* (1992b); [p]Sharrock *et al.* (1988); [q]Whitelam and Smith (1991); [r]Adamse *et al.* (1987); [s]Adamse *et al.* (1988a); [t]Ballaré *et al.* (1991); [u]López-Juez *et al.* (1990a); [v]Peters *et al.* (1991a).

simulated phototropism, the end-of-day far-red light (EOD FR) and R:FR photon ratio dependent responses in de-etiolated plants. Simulated phototropism occurs when one cotelydon of a de-etiolated seedling is covered with aluminium foil and the plant is irradiated from above with white light or R. The resulting curvature towards the uncovered cotelydon is due to R absorption by phytochrome (Shuttleworth and Black, 1977).

Other potential phytochrome-deficient mutants are: the *Arabidopsis* long-hypocotyl *(hy)*, tomato yellow-green *(yg2)*, pea long-stemmed *(lv)* and sorghum

maturity (ma_3^R) mutants. Koornneef *et al.* (1980) isolated potential phytochrome mutants in *Arabidopsis* by using the lack of hypocotyl-growth inhibition as a screening procedure. This resulted in a series of *hy* mutants at five loci *(hy1, hy2, hy3, hy4* and *hy5)*, to which another locus *(hy6)* was added by Chory *et al.* (1989a). Recently, Chory (1991) reported a seventh locus *(hy7)*, but the characteristics of this mutant have not yet been published. Although less extreme, the tomato *yg2* mutant resembles the *au* mutant (Koornneef *et al.*, 1985). Similar to the cucumber *lh* mutant, the pea *lv* mutant (Reid and Ross, 1988) has long internodes and lacks the EOD FR response (Nagatani *et al.*, 1990). Although the ma_3^R mutant was originally isolated as a non-photoperiodic mutant which flowers early, it was subsequently observed to be aberrant in phytochrome-controlled processes including anthocyanin synthesis, de-etiolation and chlorophyll content (Childs *et al.*, 1991). Similarly to the ma_3^R mutant the *Arabidopsis hy1, hy2* and *hy3* mutants also flower early and are relatively insensitive to daylength, *i.e.* non-photoperiodic (Goto *et al.*, 1991). All these mutants are deficient in the photoinhibition of hypocotyl growth and most of them show defects in greening. These data, together with their other characteristics are summarized in Table 7.2.

7.1.2. Phytochrome status in potential phytochrome mutants

The data in this thesis and in the literature have led to the hypothesis that the *au* mutant lacks the light-labile phytochrome type (PI), but not the light-stable phytochrome type(s) (PII), while in the *lh* mutant the opposite situation is the case. If this is correct, then discrete roles for these different phytochrome types have been established and can be deduced from Table 7.1, by comparing the phytochrome-regulated responses of the mutants with those of their isogenic wild types. However, before being able to conclude this, it is necessary to investigate the precise phytochrome status of the mutants. On the basis of the purification of phytochromes from D- and light-grown plants and the cloning of phytochrome *(phy)* genes, it is possible to raise monoclonal antibodies capable of distinguishing the different phytochrome types. The following terminology is used: PI for the physiologically predicted and spectrophotometrically detected light-labile phytochrome type, PHYA for the immunochemically detected protein encoded by the *phyA* gene and phy A for the photochemical active phytochrome after incorporation of functional chromophore. The relationship between the physiologically predicted and spectrophotometrically detected phytochrome types (PI and PII) and the immunochemically detected phytochrome types (PHYA, PHYB and PHYC) is only partially understood. Comparison of the amino-acid sequence of specific regions of phytochrome purified from etiolated tissue with the predicted amino-acid sequences of cloned *phy* cDNAs have established that PI is encoded by *phyA* genes (Quail, 1991). The relationship between the remaining phytochrome genes and PII has yet to be elucidated. Preliminary microsequencing data of peptides produced by proteolysis of purified pea PII (Abe *et al.*, 1989; Furuya, 1989) show that they are more similar to rice and *Arabidopsis phyB* than to *phyA* or *phyC* (Quail, 1991). However, the sequenced regions are too limited to draw a definitive

Table 7.2. Phenotypic characteristics of the *Arabidopsis hy*, tomato *yg2* and *hp*, pea *lv* and sorghum ma_3^R mutants when compared to their isogenic wild types.

	Arabidopsis						Tomato		Pea	Sorghum
Response	*hy1*	*hy2*	*hy3*	*hy4*	*hy5*	*hy6*	*yg2*	*hp*	*lv*	ma_3^R
Seed germination										
D							−[h]			
R/FR reversible promotion	+[c]	+[c]	+[c]	+[c]						
Etiolated plants										
Inhibition hypocotyl growth										
B/UV-A	+[g]	+[g]	+[g]	−[g]	−[g]	−[b]	−[f]	−[k]	−[i]	
R	−[g]	−[g]	−[g]	+[g]	−[g]			−[k]	−[i]	−[a]
FR	−[g]	−[g]	+[g]	+[g]	−[g]			−[l]	+[i]	
Anthocyanin synthesis								−[k]		−[a]
PAL activity								−[d]		
mRNA *cab* in R	−[b]	+[b]	+[m]		+[m]	−[b]				
De-etiolated Plants										
EOD FR			−[j]					+[l]	−[i]	
R:FR ratio response	+[n]	+[n]	−[n]			+[n]		+[e]		
Anthocyanin content	+[b]	+[b]				+[b]	−[h]	−[l]		−[a]
Chlorophyll content	−[b]	−[b]				−[b]	−[h]		−[i]	−[a]
Chloroplast development	−[b]	−[b]				−[b]	−[h]		−[i]	
Chloroplhyll *a*/*b* ratio	−[b]	−[b]				−[b]	−[h]		+[i]	
LHCP accumulation	−[b]	+[b]				−[b]				
mRNA *cab* and *rbcS* in WL	+[b]	+[b]				+[b]				
Leaf development	−[b]	−[b]				−[b]				

(+) response exhibited at wild-type level; (−) response different from wild type; when no data are available space is left open.
[a]Childs *et al.* (1991); [b]Chory *et al.* (1989a); [c]Cone *et al.* (1985); [d]Goud *et al.* (1991); [e]Kerckhoffs *et al.* (submitted); [f]Kerr (1981); [g]Koornneef *et al.* (1980); [h]Koornneef *et al.* (1985); [i]Nagatani *et al.* (1990); [j]Nagatani *et al.* (1991a); [k]Peters *et al.* (1989); [l]Peters *et al.* (1992a); [m]Sun and Tobin (1990); [n]Whitelam and Smith (1991).

conclusion. An analysis of the phytochrome types present in potential phytochrome mutants has been made and is summarized in Table 7.3.

7.1.2.1. The Arabidopsis hy1, hy2 and hy6 mutants. Etiolated seedlings of the *hy1*, *hy2* and *hy6* mutants contain no or dramatically reduced levels of spectrophotometrically detectable phytochrome (Koornneef *et al.*, 1980; Chory *et al.*, 1989a). Despite this, near wild-type levels of the phytochrome apoprotein are present in these mutants (Chory *et al.*, 1989a; Parks *et al.*, 1989). In the study of Parks *et al.* (1989) an antibody was used that has subsequently been shown to be selective for PHYA (Somers *et al.*, 1991), indicating that the protein data reflect the behaviour of PHYA. Since phytochrome is degraded differently *in vitro* as Pr and Pfr (Vierstra and Quail, 1982) a test employing controlled proteolysis *in vitro* can be utilized to assess the photochemical properties and the conformational structure of phytochrome. Application of this test showed that the *hy1* and *hy2* mutants display an *in vitro* proteolytic degradation pattern different from that in the wild type, which in addition, is largely unaffected by preirradiation with R or FR. Moreover, the light-regulated down-regulation of phytochrome is absent in the *hy1* and *hy2* mutants (Parks *et al.*, 1989). These data strongly suggest that the immunodetectable phytochrome (PHYA) in D-grown *hy1*- and *hy2*-mutant seedlings is largely photochemically inactive.

Restoration of the wild-type phenotype of the *hy1*, *hy2* and *hy6* mutants in white light is observed when a precursor of the phytochrome chromophore, biliverdin, is added to the growth medium (Chory, 1991; Parks and Quail, 1991). In addition, spectrophotometric and immunochemical analyses of the rescued *hy1* and *hy2* mutants demonstrate that the following parameters are indistinguishable from those in wild type: (1) photochemically active phytochrome level, (2) *in vitro* proteolysis patterns of Pr and Pfr and (3) *in vivo* light-regulated down-regulation of phy A (Parks and Quail, 1991). This reflects restoration of the photoreceptor properties and indicates that the *hy1*, *hy2* and *hy6* mutations affect chromophore biosynthesis, prior to the formation of biliverdin, rather than a phytochrome structural gene (Chory, 1991; Parks and Quail, 1991).

Assuming that all phytochrome types share the same chromophore, the mutations may lead to reduced photochemical functional levels of other phytochrome types *(e.g.* phy B and phy C) in addition to phy A. However, no definitive statement can be made about this since the photochemically active phy-B and phy-C levels have not yet been measured. Moreover, nothing is known about eventual diversity in the effectiveness of chromophore attachment to the different phytochrome types, which might result from processes such as different binding activities and assembly in different subcellular compartments.

7.1.2.2. The tomato au mutant. Whereas etiolated seedlings of the *au* mutant contain less than 5% (detection limit) of the spectrophotometrically detectable phytochrome found in wild-type seedlings, light-grown tissues of this mutant contain about 60% of the phytochrome present in the wild type (Adamse *et al.*,

Table 7.3. Analysis of phytochrome types in photomorphogenetic mutants.

Genotype	PHYA	phy A	PHYB	phy B	PHYC
Cucumber					
lh	+[i,j]	+[a,j]	−[i] / +[j]	−[a,b,j,q]	
Tomato					
au	−[h,m,n]	−[a,f,n]	+[h]	+[a,g]	
hp		+[a,p]		+[a]	
au,hp		−[p]			
yg2		−[f]			
Arabidopsis					
hy1	+[o] / −[d]	−[d,e,o]	+[l]		
hy2	+[d,o]	−[d,e,o]	+[l]		
hy3	+[d,o]	+[d,e,o]	−[l]		+[r]
hy4	+[d,o]	+[d,e,o]	+[l]		+[r]
hy5	+[d,o]	+[d,e,o]	+[l]		+[r]
hy6	−[d]	−[d]	+[l]		
Pea					
lv	+[k]	+[k]	+[k]	+[k]	
Sorghum					
ma_3^R	+[c]	+[c]	−[c]		

PHYA, PHYB and PHYC are the immunochemically dectectable proteins encoded by phytochrome genes *phyA*, *phyB* and *phyC*. They were measured by Western blotting using monoclonal antibodies. The chromophore-bearing proteins, phy A and phy B, were measured by spectrophotometry or fluorescence after formation of the Zn-chromophore complex. (+) indicates the wild-type level; (−) less than wild type or no phytochrome detectable; when no data are available space is left open.

[a]Adamse (1988); [b]Adamse *et al.* (1988a); [c]Childs *et al.* (1991); [d]Chory *et al.* (1989a); [e]Koornneef *et al.* (1980); [f]Koornneef *et al.* (1985); [g]López-Juez *et al.* (1990b); [h]López et al. (1991); [i]López-Juez *et al.* (1992); [j]Nagatani *et al.* (1989); [k]Nagatani *et al.* (1990); [l]Nagatani *et al.* 1991a; [m]Oelmüller *et al.* (1989); [n]Parks *et al.* (1987); [o]Parks *et al.* (1989); [p]Peters *et al.* (1989); [q]Peters *et al.* (1991a); [r]Somers *et al.* (1991).

1988b; López-Juez *et al.*, 1990b). Using monocotyledonous antibodies, Parks *et al.* (1987) and Oelmüller *et al.* (1989) found no immunologically detectable phytochrome in etiolated *au*-mutant tissue. However, preliminary experiments using specific antibodies against *phyA* and *phyB* gene products from dicotyledonous origin show that the PHYA polypeptide can be detected in etiolated *au*-mutant seedlings and is about 10% of that in the wild type (López *et al.*, 1991). Moreover, a comparable level of PHYB is detected in etiolated wild-type and *au*-mutant seedlings. In contrast to wild type, *in vitro* proteolysis after R and FR irradiation fails to show any conformational change in the PHYA polypeptide of the *au* mutant and light-mediated down-regulation of PHYA is absent. This suggests that etiolated *au*-mutant seedlings accumulate low levels of PHYA, which is largely photochemically inactive, and normal levels of PHYB. In light-grown wild-type and *au*-mutant seedlings comparable levels of PHYB, which is not down-regulated by light and comparable low

PHYA levels are found. These results suggest that the *au* mutant is modified with respect to the phy-A level and function, but do not give a complete explanation of the nature of the *au* mutation.

The *au* mutant produces wild-type levels of phytochrome mRNA which is fully translated *in vitro* to yield a polypeptide product of the same abundance, size and immunochemical properties as the wild-type translation product (Sharrock *et al.*, 1988). Therefore the deficiency of spectrophotometrically active phytochrome in the *au* mutant appears not to result from a lack of phytochrome gene expression, but from instability of the protein. Explanations for the phytochrome polypeptide instability *in vivo* include: (i) a phytochrome structural gene *(phyA)* mutation; (ii) an aberrant proteolytic degradation and (iii) a defect in chromophore biosynthesis or attachment to the protein (Sharrock *et al.*, 1988).

Since one phytochrome coding sequence identified by Sharrock *et al.* (1988) is located on a different chromosome to the *au* locus, the mutation probably does not affect that particular structural gene (Sharrock *et al.*, 1988). However, this phytochrome coding sequence in principle may encode a phytochrome type different from that absent in the *au* mutant. Transformation of the *au* mutant with *phyA* cDNA has so far been unsuccessful, but experiments are underway to combine, by crossings, a tomato overexpressing oat *phyA* (Boylan and Quail, 1989) with the *au* mutant (A. van Tuinen and M. Koornneef, pers. comm.).

Phytochrome degradation *via* the ubiquitin-dependent proteolytic pathway is not unique to the photoreceptor (Hatfield and Vierstra, 1990). Therefore a defect in this pathway may also affect other proteins, which results in a severely altered cellular physiology and makes it unlikely that an aberrant proteolytic degradation of phytochrome is responsible for the *au* mutation (Parks *et al.*, 1987).

Attempts to rescue the *au*-mutant with biliverdin (P.H. Quail, pers. comm.; R.P. Sharma, pers. comm.) have been unsuccesful. This result must be considered inconclusive, since a test to restore photochemical activity to phytochrome by feeding biliverdin to wild-type seedlings grown in the presence of gabaculine, an inhibitor of tetrapyrrole biosynthesis, was also unsuccessful (P.H. Quail, pers. comm.; R.P. Sharma, pers. comm.). Therefore the possibility that biliverdin is not taken up by the plant cannot be excluded. If the *au* mutant is a chromophore mutant and biliverdin is taken up into the appropriate subcellular compartment, the lesion must be either in a late chromophore biosynthetic step (biliverdin → phytochromobilin) or in the attachment of the chromophore to the phytochrome apoprotein.

7.1.2.3. The cucumber lh mutant. In Chapter 6 it was proposed that the reduction in the spectrophotometrically measured phytochrome level observed in the light-grown *lh* mutant compared to wild type arises because it is deficient in PII. Recently, López-Juez *et al.* (1992) have shown that this is indeed the case and that the *lh* mutant contains wild-type levels of PHYA, but less than 1% of PHYB. To demonstrate this López-Juez *et al.* (1992) used an antibody raised against the product of a *phyB*-gene fragment from tobacco,

which stains a band in extracts from light-grown wild-type plants, but is absent in extracts of the *lh* mutants. The nature of the *lh* mutation at the DNA level remains unknown, but the fact that it was induced by X-ray irradiation means it is possible that it is a mutant with a deletion in the *phyB* gene itself.

7.1.2.4. The Arabidopsis hy3 mutant. Similar to observations made in the *lh* mutant of cucumber, the *hy3* mutant lacks PHYB and does not display EOD FR growth responses (Nagatani *et al.*, 1991a; Somers *et al.*, 1991). Moreover, recent mapping shows that the *phyB* and *Hy3* loci are on the same chromosome and closely linked. Therefore the genetic equivalence of the *Hy3* and *phyB* loci appears likely.

In this context I have performed some preliminary experiments with a *hy* mutant which was found after *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis* Wasselewskija (WS) (Feldmann, 1991) and provided by K. Feldmann (University of Arizona, Tuscon, USA). If the inserted T-DNA disrupts a gene, this will result in a mutant phenotype that co-segregates with kanamycin resistance located on the T-DNA. At the molecular level this can be visualized by a changed restiction fragment pattern, when a genomic Southern blot is hybridized with the relevant gene probe. The hybridization of the fragment(s) present in the mutant, but absent in the wild type with both the gene probe and a T-DNA probe can be used as evidence that the gene in question is indeed disrupted.

A number of plants were selfed from the progeny of the line received from K. Feldmann, which segregates for the *hy* mutant and kanamycin resistance. The progeny of these crosses are homozygous for the *hy* mutation and all individuals are 100% resistant to kanamycin. However, in addition some wild-type plants that do not segregate *hy* mutants in their progeny were also kanamycin resistant. This indicates that the *hy* mutation is probably tagged, but that additional inserts are present. This has been observed previously in this type of material (K. Feldmann, pers. comm.). Complementation analysis showed that the *hy* mutant is allelic to *hy3* and it is therefore called a $hy3^{TAG}$ mutant. To verify if the locus involved is indeed the *phyB* locus, Southern-blot analyses were performed with *phyB* and T-DNA probes.

The T-DNA used contains the neomycin phosphotransferase II *(nptII)* gene which confers kanamycin resistance to transformed plants. Using a *nptII* probe provided by R. Weide (Department of Molecular Biology, Wageningen Agricultural University, The Netherlands) as control, a single band in the $hy3^{TAG}$ and the $hy3^{TAG}$ x WS lanes was observed (unpublished data), demonstrating that the $hy3^{TAG}$ mutant indeed contains the T-DNA insert, while the wild type does not.

Depending on the restriction sites and the position of the insert in the *phyB* gene, usage of a *phyB*-specific probe in genomic Southern-blot analyses is expected to show a band of higher molecular mass or extra bands compared to wild type. Since the sequence of *phyB* of *Arabidopsis* has been published (Sharrock and Quail, 1989), a probe for *phyB* can be prepared by the polymerase chain reaction (PCR). The probe used in these experiments was

kindly provided by Dr. N. Harberd (Cambridge Laboratory, Centre for Plant Science Research, Norwich, UK) and contains the coding region for *phyB*. As anticipated, extra band(s) appear in the $hy3^{TAG}$ lanes when the *phyB* probe is used. However, the expected disappearance of bands in the mutant was not observed.

As a probe for the right-border part of the T-DNA insert the SstII-BamHI (1 kb) fragment of the nopaline synthetase gene *(nos)* was provided by Dr. A. Depicker, Department of Genetics, University of Gent, Belgium (Depicker *et al.*, 1982). The number of bands depends on the number of inserts, but hybridization with this probe is expected to show one band in common with the same blot probed with *phyB*, since the *phyB* gene adjoins the right border of the insert. The fact that more than one band was present, indicates that more than one insert (about 3 to 4) is involved, agreeing with the genetic analysis. However, these experiments failed to provide an unequivocal answer, because the *Eco*RI blot appears to show a band in common with the extra band appearing in the $hy3^{TAG}$ lane, while the *Hind*III blot does not.

The extra band(s) appearing in the $hy3^{TAG}$ lane when the *phyB* probe was used, support the hypothesis that the *Hy3* locus is equivalent to that of *phyB*. However, the fact that no bands disappear in the $hy3^{TAG}$ lane compared to the wild-type lane and the usage of the *nos* probe does not unequivocally show a common band with the *phyB* probed blots, leaves the question unsolved. Recently, J. Chory (pers. comm.) observed evidence in support of the hypothesis that *hy3* is a mutation in the *phyB* gene by sequencing of an ethylmethane-sulphonate (EMS)-generated *hy3* mutant and comparing it with the wild-type sequence. In this manner she identified a point mutation in the *hy3* mutant. In addition, she was able to show complete genetic linkage between *Hy3* and *phyB*. Therefore the *hy3* mutant is very probably a structural *phyB*-gene mutant.

7.1.2.3. Other mutants. The sorghum ma_3^R mutant is aberrant in some phytochrome-controlled processes and appears to have normal amounts of the phytochrome (PI) dominant in etiolated tissue, but might lack one or more of the phytochromes that predominate in light-grown plant tissue (Childs *et al.*, 1991). Although this mutant requires more precise characterization, it is a candidate for a PII *(e.g.* phy B or phy C) photoreceptor mutant.

The remaining potential phytochrome mutants, the *hp*, *hy4*, *hy5* and *lv* mutants, contain normal phytochrome levels and may have defects in genes involved in the transmission of the light signal (phytochrome transduction-chain mutants) or for carrying out the appropriate developmental and physiological responses (response mutants).

7.1.3. Physiological roles of different phytochromes

The FR high irradiance reaction (HIR) is most likely mediated by PI, while on the basis of extended FR reversibility the EOD FR elongation responses can be attributed to PII (Smith and Whitelam, 1990; Chapter 5). Although it has now been established that PI is encoded by *phyA* (Quail, 1991), the relationship

between the remaining phytochrome genes and PII has not been elucidated. Therefore the question: what are the different physiological roles of distinct molecular phytochrome types can not yet be fully answered. Mutants can only help resolve the situation, when their phytochrome status and the nature of the mutation is fully characterized.

It is likely that the *Arabidopsis hy3* and the cucumber *lh* mutants are both phy B-deficient mutants. Their phenotypes show strong similarities: *e.g.* both mutants have an elongated phenotype when grown in white light and do not respond to EOD FR. The etiolated seedlings display a marked inhibition of hypocotyl elongation under continuous FR, but fail to respond strongly to continuous R (Tables 7.1 and 7.2). Moreover, both have been shown to lack the product of the *phyB* gene (PHYB), while normal PHYA levels are present (Table 7.3). Since EOD FR elongation responses are regulated by a stable phytochrome type and the *hy3* and *lh* mutants both lack the EOD FR response and PHYB, it is likely that the EOD FR response is a specific response which can be ascribed to phy B. This supports the hypothesis that the distinct phytochrome types have specific physiological functions.

The opposite situation where phy A fails to function or its action is severely depleted, appears to be found for the *Arabidopsis hy1*, *hy2*, *hy6* and the tomato *au* mutants. This line of reasoning is supported by phytochrome measurements showing that D-grown *au*-mutant seedlings lack at least 95% of the bulk light-labile pool when compared to wild type. The etiolated seedlings of the *hy1*, *hy2* and *hy6* mutants also contain no or reduced levels of detectable photoreversible phytochrome (Koornneef *et al.*, 1980; Chory *et al.*, 1989a). The PHYA found in *hy1* and *hy2* (100% when compared to wild type) and *au* (10% when compared to wild type) mutants is largely photochemically inactive. The PHYB level in the *au* mutant appears to be normal (López *et al.*, 1991) and 60% of the wild-type spectrophotometrically detectable phytochrome level was observed in light-grown *au*-mutant plant tissue. This suggests that the PHYB polypeptide is photochemically active in the *au* mutant. For the *hy1*, *hy2* and *hy6* mutants the situation concerning phy B is unclear. The fact that the phenotype of the *au*, *hy1*, *hy2* and *hy6* mutants physiologically complements that of the *lh* and *hy3* mutants in many characteristics *(i.e.* are pleiotropic for different processes) is a further indication that different phytochrome types are responsible for the regulation of different processes.

In spite of these strong indications in favour of the hypothesis that different phytochromes have different physiological roles, it is important to realize that some of the supporting evidence is either unequivocal or still unpublished. Therefore the assigment of the physiological roles played by PI (phy A) and PII (phy B) on the basis of the mutants must be considered at this stage as tentative and is summarized in Table 7.4.

Other methods applied to elucidate the putative roles of the different phytochrome types are the development of transgenic plants overexpressing foreign phytochrome genes or inhibiting the expression of individual phytochrome genes (anti-sense cDNA technique). To date, both phy-A (Keller *et*

Table 7.4. A tentative assigment of physiological roles to PI and PII.

Response	Phytochrome type responsible	
	PI	PII
Seed germination		
D	+	
R/FR reversible promotion		+
FR inhibition	+	
Etiolated plants		
Inhibition of hypocotyl growth		
R HIR	+	+
FR HIR	+	
Capacity for anthocyanin synthesis	+	
Anthocyanin synthesis		+
PAL activity		+
cab gene expression	+	
De-etiolated plants		
EOD FR		+
R:FR photon ratio response		+

al.,1989; Kay *et al.*, 1989; Boylan and Quail, 1989) and phy-B (Wagner *et al.*, 1992) overexpressors have been produced.

7.1.3.1. Seed germination. Although tomato is an example of a D-germinating species, there is evidence that the FR-absorbing form of phytochrome (Pfr) present in seeds is a prerequisite for germination. Both continuous and intermittent FR inhibits germination, while the effect of FR can be negated by R (Mancinelli *et al.*, 1966). Which type of phytochrome is responsible for these responses is unknown. In seeds, Pfr (PI and/or PII) pre-exists or can arise during hydration of phytochrome intermediates accumulated upon dehydration during the later stages of seed development on the mother plant. However, it can also be argued that upon imbibition in D, newly synthesized phytochrome (PI) becomes physiologically relevant upon conversion to Pfr during irradiation (Koornneef *et al.*, 1992). Since continuous FR can be replaced by intermittent FR to bring about inhibition, this process can be looked upon as a (prolonged) low fluence response (LFR). However, fluence-rate dependence of photoinhibition implies that a HIR is operative. Bartley and Frankland (1982) proposed a dual role of phytochrome in the photoinhibition of germination: the Pfr concentration promotes and the phytochrome cycling rate (H) inhibits the germination.

In contrast to wild type, the *au* mutant exhibits poor germination in D. It exhibits a stimulation of D germination by continuous R, but no inhibition by continuous FR (Koornneef *et al.*, 1985; Lipucci di Paola *et al.*, 1988; Georgiou and Kendrick, 1991). It was also shown that after induction of D germination by nitrate, no inhibition of germination by continuous FR was observed in the *au* mutant (Georghiou and Kendrick, 1991). The pool of phytochrome lacking in

the *au* mutant (phy A), may therefore be involved in conveying the capacity for seeds to germinate in D and in the FR inhibition of germination. The promotive effect of R on germination in the *au* mutant is FR reversible (Georghiou and Kendrick, 1991), while in the wild type the germination of FR-inhibited seed is promoted by R and the effect is reversible by FR (Mancinell, 1966). Therefore it is proposed that the R/FR reversible promotion/inhibition of germination, which is present in both wild type and mutant, is triggered by PII. The fact that the *Arabidopsis hy1* and *hy2* mutants, which are positively photoblastic, show a normal R/FR reversible promotion/inhibition of germination (Cone *et al.*, 1985) is consistent with this proposal. Since PI plays an important role in the FR HIR in de-etiolated seedlings and phytochrome plays a dual role in germination (Bartley and Frankland, 1982), the FR-HIR inhibition of seed germination may be absent in the *au* mutant, due to its PI deficiency. Dependent upon the sensitivity to Pfr this can lead to either no inhibition (Koornneef *et al.*, 1985; Georgiou and Kendrick, 1991) or stimulation (Lipucci di Paola *et al.*, 1988) of germination when compared to the germination level in D. In summary, the comparison of the wild type and *au* mutant suggests an important role for both phy A and PII in the germination process.

In both transgenic tobacco plants expressing an oat *phyA* gene and the wild type, the germination is stimulated to its maximum by white light, while the addition of FR to white light results in an inhibition of germination (McCormac *et al.*, 1991). This response could be expected on the basis of the FR-mediated HIR inhibition of germination described above. However, reduction of the FR fluence rate partially relieved the inhibition of germination in the transgenics (McCormac *et al.*, 1991). Since both lines of seeds posses similar levels of PII, but different phy-A levels, the same percentage of germination in white light may be expected, when the promotion of germination is PII-regulated. During FR addition to white light only the inhibitory effect of the FR HIR *via* phy A is expected to be observed. A lower fluence rate of the inhibitory FR results in a lower H, which in turn may release the inhibition of germination. However, the transgenic seeds have more phy A, which in contrast to the results of McCormac *et al.* (1991) is expected to result in either more or similar inhibition of germination when compared to wild type. Therefore this line of reasoning does not explain the results. McCormac *et al.* (1991) do not make a distinction between the roles of phy A and PII and presume that white light, used to promote germination, results in levels of Pfr above the saturation level for germination in both lines of the light-treated seeds. Since the higher levels of total phytochrome (Ptot) result in a higher [Pfr] in the transgenics than in wild type, a higher H is expected to be required to achieve the same degree of inhibition of germination in the transgenic seed (McCormac *et al.*, 1991). However, the higher phy-A content in the transgenics is expected to result in a higher H, which according to the results of McCormac *et al.* (1991) must be considered insufficient to fully inhibit germination, possibly due to saturation of a cycling-dependent process.

If the hypothesis that the promotion of germination is PII-regulated, while the inhibition is regulated by phy A is correct, D-germinating species which

appear to be deficient in phy B, *e.g.* the cucumber *lh* mutant, would be expected to exhibit the opposite seed germination characteristics when compared to the *au* mutant. Although no germination studies were performed with the *lh* mutant, an indication of the percentage of D germination and the FR inhibition of germination was deduced from hypocotyl-growth experiments in continuous FR described in Chapter 6. The *lh* mutant appears to exhibit normal D germination (91% and 88% for wild type and *lh* mutant, respectively), but it is not inhibited by FR (95% and 84%, respectively). Another phy B-deficient mutant, the *hy3* mutant, exhibits normal R/FR reversible promotion/inhibition of germination, although there are indications of reduced sensitivity to light (Cone *et al.*, 1985). These data are not consistent with the hypothesis that FR inhibition of seed germination is regulated by phy A and the promotion of germination is regulated by PII. However, there is an indication that germination is retarded in the *lh* mutant, suggesting that at higher fluence rates inhibition of germination would occur. Moreover, it is still unknown if PII is equivalent to phy B. If another stable phytochrome *(e.g.* phy C) plays a role in the promotion of the germination by R the response in the *hy3* mutant could be explained.

7.1.3.2. Seedling growth. The PI pool predominating in etiolated seedlings degrades after exposure to light and most likely plays an important role during the D to light transition, called de-etiolation (Kendrick and Nagatani, 1991). Despite the lack of phy A, the *au* mutant is able to de-etiolate and survive in white light, indicating that very few molecules of spectrally active phytochrome are required for plant viability. However, the B component of the white light appears to be a prerequisite to achieve de-etiolation, since under R alone the *au* mutant is lethal (Oelmüller and Kendrick, 1991). Possible explanations for this behaviour are: (i) the B/UV-A photoreceptor transduction chain overrides that of phytochrome; (ii) phy B takes over the role of phy A; (iii) the *au* mutant contains a residual amount of phy A *(i.e.* is leaky) which is sufficient for survival when the B/UV-A photoreceptor amplifies the Pfr action. The latter possibility is favoured, since both under R and B irradiation the *cab*-mRNA accumulation is under phytochrome control. However, only under B does the phytochrome become almost as effective as in the wild type (Oelmüller and Kendrick, 1991). This is consistent with the hypothesis that B stimulates the responsiveness to the residual phytochrome in the *au* mutant. Moreover, the *au,hp* double mutant shows a small but significant R/FR reversible anthocyanin accumulation after a B pretreatment (Chapter 2), indicating that there is active residual phytochrome present.

The HIR inhibition of hypocotyl growth in etiolated and de-etiolated seedlings reported by Beggs *et al.* (1980) suggests the existence of two seperate photoreceptors, one responsible for the FR and B effects and one for the R effect. They showed that continuous FR and B was very inhibitory in D-grown seedlings with a high Ptot level, whereas a R pulse, which reduces the Ptot *via* phy A destruction, resulted in a reduction of this inhibition. In light-grown plants the effectiveness of FR and B almost disappears, but the inhibition of

the hypocotyl growth by continuous R remains unchanged. These results imply that a light-stable phytochrome could mediate the R component, while a light-labile phytochrome regulates the FR component of the HIR. Since the R HIR is absent in the phy B-deficient *lh* and *hy3* mutants, phy B might be the stable phytochrome pool responsible for the R component, while phy A, which is present at normal levels in both mutants, probably mediates the FR component of the HIR (Somers *et al.*, 1991).

In contrast to wild type, transgenic tobacco seedlings overexpressing an oat *phyA* gene retained the FR-mediated inhibition of hypocotyl extension after 2 d of de-etiolation in white light (McCormac *et al.*, 1991). This may reflect the persistence of a FR HIR mediated by the high levels of oat phy A and is an extra indication that phy A functions in the FR HIR.

The phy A-deficient *hy1*, *hy2* and *au* mutants not only lack the FR HIR, but also the R HIR. Possibly both PI and PII are involved in the R HIR. Mutants deficient in phy A (*e.g.* the *au* mutant) or phy B (*e.g.* the *lh* and *hy3* mutants) both exhibit long hypocotyls, while overexpression of the *phyA*, as well as the *phyB* gene causes hypocotyl inhibition. This indicates that neither phy A nor phy B alone is sufficient to control normal hypocotyl extension. Somers *et al.* (1991) therefore proposed that the two phytochromes initiate the same or seperate transduction chains in which the degree of hypocotyl growth in wild-type plants is dependent on the levels of both the phy A and phy B established in the light. Not all phy A overexpressors have pronounced short phenotypes. Tobacco plants overexpressing the rice *phyA* gene only show short hypocotyls under low fluence rates of R at the time of de-etiolation (Nagatani *et al.*, 1991b), indicating that these plants show increased sensitivity to light at this developmental stage. Koornneef *et al.* (1992) suggested that the relatively large PI pool normally present in D-grown seedlings acts as an antenna and allows the plants to de-etiolate quickly and completely upon reaching the light environment. All these examples are physiological indications that the relatively large amount of phytochrome present at the moment of transition from D to light is a mechanism for increasing sensitivity to light at this crucial stage of development of a seed plant. During de-etiolation the PI level declines by Pfr destruction and down-regulation of its own gene until a low steady-state level is reached and similar amounts of PI and PII are present (Abe *et al.*, 1985). While the PII pool appears to become more important upon de-etiolation, PI might continue to play a role. Since plants naturally grow in light/D cycles they re-etiolate each night and de-etiolate each day, especially if we consider the young developing tissues at the apex (Kendrick and Nagatani, 1991). It is proposed that PII plays a dominant role in the regulation of the elongation growth in light-grown plants (Smith and Whitelam, 1990; Koornneef *et al.*, 1992). McCormac *et al.* (1991) demonstrated that phy A must be removed during de-etiolation in order that PII can operate effectively in shade-avoidance responses (also see 7.1.3.5). In cucumber, the transition in function from PI to PII may already occur during hypocotyl growth (Chapter 6), while in tomato the PII action is less at the hypocotyl stage (Koornneef *et al.*, 1992).

7.1.3.3. Anthocyanin synthesis. Anthocyanin content is under the control of phytochrome in the wild type and is severely deficient or fails to accumulate in the *au* mutant. Since there is a correlation with the absence of phy A, this pool is proposed to play an important role in anthocyanin accumulation (Chapters 2 and 3). However, escape experiments with the *hp* mutant show that the Pfr regulating the anthocyanin synthesis must be relatively stable (Chapter 4) and it is concluded that the capacity for anthocyanin synthesis is established by PI, while the anthocyanin synthesis is actually photoregulated *via* a stable phytochrome type. The finding that the potentially PII-deficient ma_3^R mutant also lacks the phytochrome regulation of anthocyanin synthesis in both etiolated and de-etiolated plants supports this conclusion.

Phenylalanine ammonia lyase (PAL) is an important enzyme in flavonoid biosynthesis and its activity is a prerequisite for anthocyanin synthesis. Similarly to anthocyanin, PAL activity in tomato seedlings is regulated *via* phytochrome (Goud *et al.*, 1991). However, comparison of anthocyanin accumulation and PAL photoinduction revealed that phytochrome-mediated induction of PAL and anthocyanin are not correlated (Goud *et al.*, 1991). They found that in R the *au*- and *au,hp*-mutant seedlings show a similar increase in PAL level to that observed in the wild type, whereas there is little formation of anthocyanin in these mutants. This demonstrates that despite the deficiency of the bulk labile phytochrome pool, the photoregulation of PAL is not impaired in the *au* and *au,hp* mutants and suggests that PII plays a role in the regulation of PAL.

7.1.3.4. Greening. Since the *hy1*, *hy2*, *hy6* and *au* mutants show characteristics such as strongly reduced chlorophyll content, less well-developed chloroplasts and reduced *cab* expression, it can be postulated that PI plays an important role in the greening process. However, an alternative possibility is that the mutation simultaneously alters two independent processes: PI synthesis and greening. This could arise in a mutant not modified with regard to some aspect of chromophore biosynthesis, since phytochrome and chlorophyll both require tetrapyrrole skeletons for their chromophores. The observed accumulation of near wild-type levels of PHYB in light-grown *au* mutants, while PHYA is extremely reduced (López *et al.*, 1991), could be explained by the chromophore availability increasing after de-etiolation. Changes in the environmental conditions can alter the degree of greening in light-grown *au* mutants, *e.g.* greening appears to be stronger under low fluence-rate than under high fluence-rate light. This indicates that the *au* mutant is capable of producing extra chlorophyll and argues against a limitation in tetrapyrrole availability. Since the chlorophyll production requires relatively large amounts of tetrapyrrole precursors compared to the requirement for phytochrome synthesis, the latter process would be expected to be saturated well before that of chlorophyll synthesis. It is therefore proposed that the aberrant greening process in the *hy1*, *hy2*, *hy6* and *au* mutants is a result of phytochrome deficiency.

The biosynthesis of chloroplasts requires light and involves the synthesis and accumulation of numerous protein components of the photosynthetic apparatus. The mRNA levels of genes involved *(e.g. cab, rbcS)* are regulated by light *via* phytochrome (Thompson, 1991). Although the development of leaves and chloroplasts in the *hy1*, *hy2*, *hy6* and *au* mutants is different from that in wild type (Koornneef *et al.*, 1985; Chory *et al.*, 1989a), the expression of several light-regulated genes involved in the greening process is not aberrant from that of wild type at high fluence rates (Chory *et al.*, 1989a). However, when etiolated plants are subjected to inductive pulses of R, instead of high fluence-rate white light, the mutants are defective in the phytochrome regulation of *cab* genes (Sharrock *et al.*, 1988; Chory *et al.*, 1989a). Since the small induction of the *au*-mutant *cab* mRNA by R is still reversible by FR, the residual amount of *cab* mRNA accumulated in the mutants is more probably due to residual Pfr, than to action of a different transduction chain. Far-red light alone induces a significant increase in *cab* mRNA in wild type but not in the *au* mutant, indicating that the mutant has not retained the very low fluence phytochrome response (VLFR) (Sharrock *et al.*, 1988). Ken-Dror and Horwitz (1990) came to the same conclusion on the basis of FR pulse experiments and fluence-response curves for potentiation of greening by a R pulse.

These data suggest that in light-grown *Arabidopsis* plants far fewer molecules of the phytochrome type lacking in the mutants are required for wild-type gene expression than in D-grown plants. Possibly, the PI pool plays a dominant role in the regulation of gene expression in D-grown plants, while in light-grown plants this phytochrome pool plays a minor role and PII takes over this function (Chory, 1991). Although photoregulated genes are expressed at wild-type levels in PI-deficient mutants when grown in white light, the mutants still display aberrant properties associated with greening: *e.g.* reduced chlorophyll content, higher chlorophyl *a/b* ratios, smaller leaves and lower levels of LHCPs. Chory *et al.* (1989a) proposed that the primary role for phytochrome in green plants is to modulate the degree of chloroplast development rather than to trigger only the initiation of chloroplast development. In contrast, in etiolated plants phytochrome has been proposed to initiate chloroplast development (Ken-Dror and Horwitz, 1990).

7.1.3.5. Shade-avoidance responses. Supplementary FR to white light simulates the presence of vegetation and initiates the shade-avoidance syndrome (Smith, 1986). In *Arabidopsis*, which is a rosette plant, the shade-avoidance elongation response is expressed by longer petioles (Nagatani *et al.*, 1991), while in cucumber and tomato it also results in longer internodes (López-Juez *et al.*, 1990a). Both the *lh* and *hy3* mutants lack the EOD FR elongation response, while the *au* mutant exhibits this response. The response to supplementary FR is present in the *hy1*, *hy2*, *hy6* and *au* mutants, but severely reduced in the *lh* and *hy3* mutants. Whitelam and Smith (1991) have shown that the response to supplementary FR in the *lh* and *hy3* mutants is not completely absent. If this is the case, it indicates that light-grown seedlings of these phy B-deficient mutants contain some phytochrome that can function in the shade-avoidance

responses. Therefore, either these mutants are leaky and still possess a minor phy B pool or one or more other phytochrome types are functional to a minor extent in R:FR ratio perception. In any case phy B appears to play the dominant role in vegetational-shade detection.

Although transgenic tobacco overexpressing an oat *phyA* gene showed a normal EOD FR response (Smith *et al.*, 1991), no increases in extension growth under low R:FR treatments were shown and at low fluence rates growth extension rates were even significantly decreased by low R:FR (McCormac *et al.*, 1991). A possible explanation is that the persistence of phy A in light-grown plants facilitates the continuation of the response normally observed in etiolated seedlings during de-etiolation (McCormac *et al.*, 1991). Such a FR HIR would operate antagonistically to the normal stimulation of extension growth by supplementary FR and might therefore nullify or even reverse the normal shade-avoidance response. This suggests that phy A is normally removed during de-etiolation in order that shade-avoidance responses can operate effectively (McCormac *et al.*, 1991).

On the basis of this discussion it is proposed that phy B functions as a photoreceptor for vegetational shade and neighbour detection.

7.1.3.6. Conclusions. In conclusion, the phytochrome type responsible for shade-avoidance responses appears to be phy B. In the other processes discussed, including seed germination, seedling growth and anthocyanin synthesis, both phytochrome types appear to play a role. The difference in light stability of phy A and phy B might be an indication of the role played by the different phytochrome types at different developmental stages of the plant. The relatively large amount of phytochrome present at the moment of transition from D to light is a possible mechanism for increasing sensitivity to light at this crucial stage of development of a seed plant. During de-etiolation the phy-A level declines by Pfr destruction and down-regulation of its own gene until a low steady-state level is reached consisting of similar amounts of phy A and phy B. Although the phy B pool appears to become important upon de-etiolation, phy A might continue to play a role in processes such as sustaining chloroplast development.

7.2. TRANSDUCTION CHAIN MUTANTS

If PI and PII have discrete functions there must be some difference concerning their signal transduction pathways. Different phytochrome types may work *via*: entirely distinct pathways; seperate branches of a common signal transduction pathway or identical pathways. In the latter two situations compartmentalization, temporal seperation and tissue specificity may play a role in deciding which phytochrome type uses the transduction chain. The phytochrome signal transduction chain(s) is (are) still unclear and a genetic approach could help to unravel it (them).

The *hp* mutant of tomato exhibits exaggerated phytochrome responses, whereas the phytochrome content and the characteristics of the phytochrome system are similar to that in wild type. Therefore the *hp* mutant is not a photoreceptor mutant. In contrast to wild type, the *hp* mutant does not require coaction of the B photoreceptor and phytochrome for normal development and exhibits maximum anthocyanin synthesis and hypocotyl growth in R alone *i.e.* it mimics the action of B. On the basis of its recessive (loss-of-function) nature it is proposed that the phytochrome action in D-grown seedlings is under the constraint of the *Hp*-gene product (HP) (Chapter 4). Both exposure to B and the *hp* mutation appear to result in reduction of HP or its effectiveness. From this description it may be deduced that the *hp* mutant is a mutation which affects an amplification step in the phytochrome transduction chain. It is proposed that the amplification only affects phy-A action, since: (i) the phy A-lacking *au,hp* double mutant shows severely reduced or no anthocyanin accumulation; (ii) the overexpression of *phyA* exhibits similar exaggerated phytochrome responses (Boylan and Quail, 1989) and (iii) phy B-related shade-avoidance responses are normal in the *hp* mutant when compared to wild type. This suggests that there are indeed different transduction chains for phy A and phy B. However, it remains possible that the phy-B transduction chain in the case of the shade-avoidance response is already saturated. The photoregulation of PAL activity does show a higher level in the *hp* mutant when compared to the *au*-, *au,hp*-mutant and wild-type level (Goud *et al.*, 1991). If the PAL activity is indeed regulated by PII as proposed in Table 7.4, this suggests that amplification of a phy-B transduction chain is also possible.

Other potential transduction chain mutants are de-etiolation *(det)* (Chory *et al.*, 1989b; Chory and Peto, 1990) and constitutive photomorphogenesis *(cop)* (Deng *et al.*, 1991) mutants, which de-etiolate in D. On the basis of the recessive (loss-of-function) nature of these mutants it was proposed that de-etiolation is prevented in D-grown seedlings by the *Det*-gene product (DET) and that Pfr leads to its depletion or inhibits its action during normal de-etiolation. There are 4 to 5 complementation groups of these *det* mutants (Chory, 1991) and the phenotype of the *det1,det2* double mutant is additive (Chory *et al.*, 1991). This implies that DET1 and DET2 do not interact and that they act in entirely distinct pathways or seperate branches of a common signal transduction pathway downstream from Pfr. Although the hypocotyl length of D-grown *hp*-mutant seedlings is significantly reduced when compared to wild type (Chapter 3), the *hp* mutant, unlike the *det* mutants, does not show other phenotypic features characteristic of de-etiolation. However, both *hp* and *det* mutants are aberrant concerning a regulatory molecule exerting negative control over the light response.

The *Arabidopsis hy4* mutant shows a defective inhibition of the hypocotyl growth in B (Koornneef *et al.*, 1980). Although it is possible to hypothesize a defect related to the B photoreceptor, this is unlikely because several known B responses are normal in this mutant (Chory, 1991). The *hy4* mutant is not allelic to the blue-light uninhibited *(blu)* mutants isolated by Liscum and Hangarter (1991).

The *Arabidopsis hy5* mutant is a potential transduction-chain mutant, but, to date, experiments have failed to elucidate its molecular basis. The *cab* genes in the *hy5* mutant are expressed normally with respect to phytochrome regulation, suggesting that the genetic defect does not affect steps in the signal transduction pathway leading to the phytochrome-regulated expression of *cab* genes (Sun and Tobin, 1991). The *hy5* mutant may therefore be a response mutant affected only in the hypocotyl growth, although it is still possible that there is a defect in a downstream branch of the pathway or another pathway not affecting *cab* gene expression. Since the *hy5* mutation in combination with other *hy* mutations had an additive effect on the inhibition of hypocotyl elongation (Koornneef *et al.*, 1980; Chory, 1991), the different *Hy*-gene products do not interact and affect the hypocotyl growth in distinct ways.

No differences were found between the pea long-stemmed *(lv)* mutant and its isogenic wild type concerning spectrophotometrically and immunochemically determined phytochrome levels in D- and light-grown plant tissue (Nagatani *et al.*, 1990). Nevertheless, the *lv* mutant showed reduced inhibition of shoot length in continuous R when compared to wild type, while no difference between the mutant and wild type was found when plants were grown in continuous D and FR (Nagatani *et al.*, 1990). These observations together with the lack of the EOD FR elongation response resemble those of the *lh* and *hy3* mutants, both shown to lack PHYB. Therefore the *lv* mutant is a candidate for a phy-B transduction-chain mutant.

Although a mutation in the photoreceptor of a specific phytochrome type can not in principle be seperated from a mutation in an early step of its transduction chain, in recent years methods have been developed which make it possible to detect the presence or absence of specific phytochrome types. Most mutants selected *via* classical mutagenesis have turned out to be photoreceptor mutants and no candidates for a mutant in early steps of the signal transduction have been found. When the transduction chains proposed in the literature (Quail,1991) involve general mechanisms such as: the phosphatidyl-inositol pathway, Ca^{2+} and calmodulin as second messengers, it can be predicted that many essential processes are affected. Therefore the likelyhood that such mutations are lethal is almost inevitable.

More direct genetic approaches have also been applied in addition to classical mutagenesis in an attempt to generate mutants. An example is the use of 'suicide genes' which involve the fusion of a phytochrome-regulated promotor from a *cab* gene to a selectable reporter gene. Different bacterial genes encoding products which are lethal for plants have been used as reporter genes (Altschmied and Chory, 1990; Karlin-Neumann *et al.*, 1991). After transformation with such a construct, only mutants in which the phytochrome-regulated transcriptional response is affected can survive.

Although current evidence supports the existence of different pathways or at least different branches of a common pathway, the complexity of phytochrome signal transduction is far from being fully elucidated. Further investigation of the mutants characterized in this thesis, the search for new mutants and cloning of their modified genes will play an essential role in achieving this goal.

SUMMARY

Light is an important environmental factor controlling plant growth and development. It is not only used as a source of energy for photosynthesis, but also as a signal to detect information about the environment for photomorphogenesis. Photomorphogenesis is the subject of this thesis. In order to detect parameters of the light environment such as quality, quantity, direction and duration at least three different photoreceptors have evolved. They include the chromoprotein phytochrome, a blue light (B)/UV-A photoreceptor and a UV-B photoreceptor. Phytochrome is the best characterized photomorphogenetic photoreceptor and absorbs primarily in the red light (R) and far-red light (FR) regions of the spectrum. It exists in two forms: the R-absorbing (Pr) and the FR-absorbing (Pfr) form, which are interconvertible by R and FR, respectively. The active form of phytochrome, Pfr, *via* a chain of events (transduction chain), controls processes such as: germination, de-etiolation, elongation growth, gene expression and flowering. Physiological, spectrophotometrical and immunochemical evidence has accumulated which indicates that multiple types of phytochrome exist. Two phytochrome types are distinguished at the physiological and spectrophotometrical level: (i) abundant in dark (D)-grown seedlings and decreasing rapidly in the light upon conversion to Pfr (the light-labile type or PI); (ii) relatively stable as Pfr and present in D-grown seedlings and in light-grown plants (the light-stable type or PII). The existence of these different phytochrome types has been confirmed with the aid of antibodies and they have been shown to be products of different phytochrome genes *(e.g. phyA, B* and *C).* The following terminology has been adopted in this thesis: PI for the physiologically predicted and spectrophotometrically detected light-labile phytochrome type, PHYA for the immunochemically detected protein encoded by the *phyA* gene and phy A for the photochemically active phytochrome after incorporation of the functional chromophore. The relationship between the physiologically predicted and spectrophotometrically detected phytochrome types (PI and PII) and the immunochemically detected phytochrome types (PHYA, PHYB and PHYC) is only partially understood. Comparison of the amino-acid sequence of specific regions of phytochrome purified from etiolated tissue with predicted amino-acid sequences of cloned *phy* cDNAs have established that PI is encoded by *phyA* genes. The relationship between the remaining phytochrome genes and PII has yet to be elucidated. Preliminary microsequencing data of specific regions of purified pea PII show more similarity to rice and *Arabidopsis phyB* than to *phyA* and *phyC.*

The roles of the different phytochromes. Since a family of genes encodes different molecular types of phytochrome, it is conceivable that these types have different physiological roles. In this thesis this concept has been elaborated and an attempt has been made to determine the roles played by the different members of the phytochrome family (Chapter 7). For this purpose

phytochrome mutants were used. The comparison of the response of a mutant affected in the abundance or function of a particular phytochrome type with that of its isogenic wild type should show directly the relevance of the phytochrome type concerned. As putative phytochrome mutants the tomato *aurea (au)* and the cucumber long-hypocotyl *(lh)* mutants were used. A number of phytochrome-regulated processes were measured to characterize these mutants: (i) anthocyanin synthesis; (ii) inhibition of the hypocotyl growth; (iii) chlorophyll content; (iv) end-of-day (EOD) FR response.

In contrast to the wild type, etiolated tomato *au*-mutant seedlings exhibit no anthocyanin accumulation after a 12 h B pretreatment terminated with a saturating R pulse (Chapter 2) or after 5 d broad-band UV-A, B or R (Chapter 3). Fluence rate-response relationships for anthocyanin synthesis after 24 h irradiation with different wavelengths also reveal that *au*-mutant seedlings do not accumulate anthocyanin (Chapter 4). The fact that young de-etiolated hypocotyls of the *au* mutant show some anthocyanin accumulation after 4 to 5 d white light/D cycles (Chapter 3), suggests that either a B photoreceptor or phytochrome eventually becomes functional. However, in leaves of adult light-grown *au*-mutant plants no anthocyanin is detectable (Chapter 5). These data indicate that the *au* mutant has a severe deficiency in capacity for anthocyanin synthesis and that the phytochrome type lacking in the *au* mutant plays an important role in the regulation of anthocyanin synthesis. Another phytochrome-regulated process, the inhibition of hypocotyl growth, also appears to be affected in the *au* mutant. Two experiments: (i) a 5 d exposure to UV-A, B or R (Chapter 3) and (ii) fluence rate-response relationships for the increase in hypocotyl growth during a 24 h light period of different wavelengths (Chapter 4) reveal that only B and UV-A are effective in bringing about hypocotyl-growth inhibition of the *au* mutant. This suggests that the phytochrome type lacking in the *au* mutant plays an important role in the regulation of the inhibition of hypocotyl growth. The fact that only B and UV-A are effective implies that a photoreceptor, apart from phytochrome is active at these wavelengths. However, B is not as effective in inducing hypocotyl-growth inhibition of the *au*-mutant as wild-type seedlings (Chapters 2 and 3). Tomato *au*-mutant plants grown in the greenhouse under natural light/D cycles show only slightly longer internodes than those of the wild-type plants (Chapter 5). Moreover, the capacity to react to an EOD FR pulse is maintained in the *au* mutant (Chapter 5). The EOD FR treatment is a method of triggering a phytochrome-mediated shade-avoidance-like response, which results in the promotion of stem elongation in order to avoid vegetational shade. The perception of an increased proportion of FR occuring in canopy shade light is a specific function of phytochrome in light-grown plants. Finally, *au*-mutant plants remain yellow-green throughout their life due to reduced chlorophyll levels (Chapter 5).

The hypocotyl-growth rate of the cucumber *lh* mutant and its isogenic wild type is the same in D and is equally inhibited by continuous FR. However, under continuous R, and to a lesser extent B, the inhibition is dramatically less in the *lh* mutant (Chapter 6). *In vivo* spectrophotometric phytochrome measurements show that etiolated *lh*-mutant and wild-type hypocotyl hooks

contain similar amounts of phytochrome, while after 4 d FR, R and B, less phytochrome is detected in the mutant than the wild type. Since the rate of destruction and resynthesis of PI is identical in the wild type and *lh* mutant, it has been proposed that the *lh* mutant exhibits a reduced phytochrome function in light-grown plants due to depletion of a stable phytochrome type (Chapter 6).

In order to determine the role played by the phytochrome type deficient in a particular mutant not only a detailed investigation of the characteristics, but also knowledge of the phytochrome status of the mutant is necessary. Therefore the results summarized above are integrated and reviewed in the light of relevant literature (Chapter 7). This has led to the hypothesis that the *au* mutant lacks phy A, but not phy B, while in the *lh* mutant the opposite situation is the case. If this is correct, then discrete roles for different molecular phytochrome types have been established and can be deduced by comparing the phytochrome-regulated responses of the mutants with those of their isogenic wild types. In addition to the *au* and *lh* mutants, the characteristics and phytochrome status of other phytochrome mutants are described and on the basis of these data an attempt is made to assign specific functions to the different phytochrome types (Chapter 7). The phytochrome type responsible for shade-avoidance responses appears to be phy B. In the other processes discussed, including seed germination, seedling growth and anthocyanin synthesis, both phytochrome types appear to play a role. The difference in light stability of phy A and phy B might be an indication of the role played by the different phytochrome types at different developmental stages of the plant. Possibly the relatively large amount of phytochrome present at the moment of transition from D to light is a mechanism for increasing sensitivity to light at this crucial stage of development of a seed plant. During de-etiolation the phy-A level declines by Pfr destruction and down-regulation of its own gene until a low steady-state level is reached consisting of similar amounts of phy A and phy B. Although the phy B pool appears to become important upon de-etiolation, phy A might continue to play a role in processes such as sustaining chloroplast development.

Transduction chain mutants. If the different phytochrome types have discrete functions there must be some difference concerning their signal transduction pathways. However, the phytochrome signal transduction chain(s) is (are) unclear and a genetic approach could help to unravel them. In this thesis a potential phytochrome transduction chain mutant, the high pigment *(hp)* mutant of tomato, has been studied.

In contrast to wild type, the *hp* mutant demonstrates detectable phytochrome control of anthocyanin synthesis in response to a single R pulse (Chapter 2). After a 12 h B pretreatment the phytochrome-regulated anthocyanin accumulation is 4 to 8-fold higher in the *hp* mutant when compared to the wild type (Chapters 2 and 3). Although the *au* mutant does not accumulate anthocyanin after 12 h B terminated with a R pulse, the *au,hp* double mutant shows a small (3% of the *hp* mutant) R/FR reversible response (Chapter 2). In contrast to wild type, 12 h B can be replaced by a 12 h R pretreatment in the

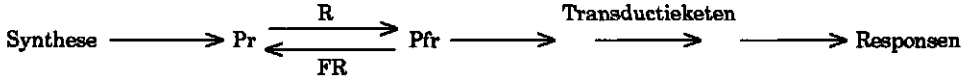
hp mutant, both resulting in maximal phytochrome-regulated anthocyanin synthesis (Chapter 3). Fluence rate-response relationships for anthocyanin synthesis after 24 h irradiation with different wavelengths reveal that the *hp* mutant exhibits high anthocyanin accumulation under all wavelengths studied. Especially the strong response observed under green light, which is inefficient in phytochrome cycling, is noteworthy (Chapter 4). However, adult light-grown *hp*-mutant plants appear to accumulate similar amounts of anthocyanin to wild type (Chapter 5). These data show that the *hp* mutant, in contrast to the *au* mutant, exhibits enhanced levels of anthocyanin when compared to wild type. Another phytochrome-regulated process, the inhibition of hypocotyl growth, is also affected in the *hp* mutant. After a 5 d exposure to UV-A, B or R the hypocotyl growth of the *hp* mutant is strongly inhibited, while, in contrast to wild type, R is almost as effective as B. Under these light conditions the hypocotyls of the *au,hp* double mutant are more inhibited than those of the *au* mutant (Chapter 3). Fluence rate-response relationships for the increase in hypocotyl growth during a 24 h irradiation with different wavelengths also show that the *hp* mutant has the shortest hypocotyls under all wavelenths studied. Adult light-grown *hp*-mutant plants display a normal EOD FR response resulting in an increase in elongation growth and a reduction of anthocyanin accumulation (Chapter 5). Experiments performed in order to explain the *hp*-mutant phenotype reveal that: (i) the higher responsiveness of the *hp* mutant is not caused by a higher absolute [Pfr] or a reduced rate of phytochrome destruction, (ii) the *hp* mutant does not convey hypersensitivity to Pfr; (iii) the kinetics of anthocyanin accumulation in continuous R are similar in the wild type and mutant (Chapter 4). Escape from FR reversibility shows that the anthocyanin synthesis after different light pretreatments terminated with a R pulse is still 50% FR reversible after about 5 h D in both the wild type and *hp* mutant (Chapter 4).

In conclusion, the *hp* mutant exhibits exaggerated phytochrome responses, whereas the phytochrome content and the characteristics of the phytochrome system are similar to those in the wild type. Therefore the *hp* mutant is not a photoreceptor mutant. In contrast to the wild type, the *hp* mutant does not require the coaction of the B photoreceptor and phytochrome to exhibit normal development and maximum anthocyanin synthesis and hypocotyl growth in R alone. In other words, it mimics the action of B. On the basis of its recessive (loss-of-function) nature it is proposed that the phytochrome action in D-grown seedlings is under the constraint of the *Hp*-gene product (HP). Both exposure to B and the *hp* mutation appear to reduce HP or its effectiveness. From this description it may be deduced that the *hp* mutant is a mutation which affects an amplification step in the phytochrome transduction chain. It is proposed that the amplification only affects phy-A action, since: (i) the phy A-lacking *au,hp* double mutant shows severely reduced or no anthocyanin accumulation; (ii) the overexpression of *phyA* exhibits exaggerated phytochrome responses and (iii) phy B-related shade-avoidance responses are normal in the *hp* mutant compared to the wild type. Although a few other transduction-chain mutants have been isolated (Chapter 7), the complexity of the phytochrome signal

transduction is far from being elucidated. Further investigation of the mutants characterized in this thesis, the selection of new mutants and cloning of their modified genes will play an essential role in achieving this goal.

SAMENVATTING

Hogere planten hebben het vermogen hun groei en ontwikkeling aan te passen aan de omgeving waarin ze zich bevinden. Externe factoren zoals zwaartekracht, water, temperatuur en licht spelen hierbij een sturende rol. Licht is voor de plant niet alleen van belang als energiebron voor de fotosynthese, maar ook als informatiebron voor de fotomorfogenese. Fotomorfogenese - het proces waarbij de groei en ontwikkeling van de plant worden afgestemd op het voor de plant beschikbare licht - is onderwerp van dit proefschrift. Om licht te kunnen waarnemen, beschikken planten over pigmenten, ook wel fotoreceptoren genoemd. Voor wat betreft de fotomorfogenese zijn een aantal fotoreceptoren bekend: (i) fytochroom, vnl. werkzaam in rood en ver-rood licht; (ii) blauw licht en UV-A absorberende fotoreceptor(en); (iii) een UV-B absorberende fotoreceptor. Fytochroom is als enige geïsoleerd en gezuiverd. Het is een blauwgroen pigment bestaande uit een eiwit met een chromofoorgroep. Deze groep is opgebouwd uit vier pyrrolkernen en is het deel van fytochroom dat absorptie van licht mogelijk maakt. Fytochroom komt voor in twee vormen: de rood licht (R) absorberende vorm (Pr) en de ver-rood licht (FR) absorberende vorm (Pfr) (zie onderstaand schema). De Pr wordt gesynthetiseerd in het donker en wordt omgezet in Pfr na R belichting. Door FR belichting wordt Pfr weer omgezet in Pr. De vorming van Pfr leidt, via een keten van reacties (transductieketen), tot een groot aantal uiteenlopende fysiologische responsen zoals: kieming, deëtiolatie, genexpressie en bloei. Deëtiolatie vindt plaats wanneer in donker opgegroeide (geëtioleerde) planten aan licht worden blootgesteld (zie Figuur 1.1 op bladzijde 2). Geëtioleerde planten groeien snel, hebben nauwelijks kleur en de kleine samengevouwen bladeren zijn nog naar beneden gericht. Gedeëtioleerde planten worden daarentegen gekenmerkt door remming van de lengtegroei, het uitvouwen van de bladeren en de aanmaak van chlorofyl en andere pigmenten, bijvoorbeeld anthocyaan. Bovengenoemde responsen worden gestimuleerd door een R puls en geremd door een FR puls. Wanneer na een R puls een FR puls wordt gegeven, wordt de stimulerende werking van R opgeheven. Een response die aan deze voorwaarde voldoet wordt R/FR omkeerbaar genoemd. De R/FR omkeerbaarheidstest wordt gebruikt om aan te tonen of een respons door fytochroom gereguleerd wordt.



Sinds kort zijn er bewijzen voor het bestaan van meerdere typen fytochroom. Met behulp van fysiologische proeven en spectrofotometrie kunnen de volgende typen onderscheiden worden: het in licht labiele fytochroom (PI) en het in licht stabiele fytochroom (PII). In geëtioleerde planten is voornamelijk PI aanwezig dat bij de overgang naar licht wordt afgebroken tot een laag (evenwichts)-

niveau. Het PII is zowel in geëtioleerde als gedeëtioleerde planten op een laag niveau aanwezig en wordt niet afgebroken door het licht. Het bestaan van deze fytochroomtypen werd bevestigd met behulp van specifieke antilichamen. Toepassing van moleculair biologische techieken maakte duidelijk dat de verschillende fytochroomtypen producten van verschillende fytochroomgenen (bv. *phyA, B, C* etc.) zijn. In dit proefschrift wordt de volgende terminologie gebruikt: PI voor de op fysiologische en spectrofotometrisch niveau aantoonbare in licht labiele vorm van fytochroom; PHYA voor het met behulp van antilichamen aantoonbare produkt (eiwit) van het *phyA*-gen en phy A voor het fotochemisch actieve fytochroom na incorporatie van de chromofoor. De relatie tussen de fysiologisch en spectrofotometrisch aantoonbare (PI en PII) en de met behulp van antilichamen te onderscheiden (PHYA, PHYB en PHYC) fytochroomtypen wordt slechts ten dele begrepen. Vergelijking van de aminozuurvolgorde van het fytochroomeiwit van geëtioleerde planten met de voorspelde aminozuurvolgorde van fytochroomgenen laat zien dat het *phyA*-gen codeert voor PI. Hoewel er een zelfde relatie lijkt te zijn tussen PII en *phyB* zijn de bewijzen nog erg miniem.

Probleemstelling en -aanpak. Het bestaan van verschillende typen fytochroom heeft geleid tot de hypothese dat elk fytochroomtype zijn eigen fysiologische taak heeft. Een deel van dit proefschrift is gewijd aan deze hypothese. Er werd voor een genetische benadering van het probleem gekozen door te werken met mutanten, die een defect in de fotomorfogenese vertonen. Deze fotomorfogenetisch mutanten kunnen worden onderverdeeld in: fotoreceptor-, transductieketen- en responsmutanten. Het belang van het gemuteerde deel wordt onmiddellijk duidelijk wanneer de mutant wordt vergeleken met zijn wildtype (normale plant). De vraag of verschillende typen fytochroom verschillende functies hebben, kan beantwoord worden door te werken met mutanten waarin een specifiek fytochroomtype ontbreekt (fytochroommutanten).

Een ander probleem in de fotomorfogenese is het ontbreken van kennis over grote delen van de transductieketen. Transductieketenmutanten zouden het inzicht kunnen vergroten. Daarom wordt in een ander deel van dit proefschrift een potentiële transductieketenmutant uitvoerig gekarakteriseerd.

De taken van de verschillende fytochroomtypen. Om te bepalen of de verschillende fytochroomtypen verschillende taken hebben, werden twee fytochroommutanten gebruikt: de *aurea (au)* mutant van tomaat en de lange hypocotyl *(lh)* mutant van komkommer. Er werd een aantal door fytochroom gereguleerde processen gemeten om deze mutanten te karakteriseren: (i) de synthese van het roodpaarse pigment, anthocyaan; (ii) de remming van de hypocotyllengtegroei; (iii) het chlorofylgehalte; (iv) de eind-van-de-dag FR respons. De resultaten van de uitgevoerde experimenten staan beschreven in de hoofdstukken 2 tot en met 6 en worden hieronder samengevat.

In tegenstelling tot wildtype zaailingen vindt er in geëtioleerde zaailingen van de *au* mutant na korte (maximaal 24 uur) belichtingsperioden met

verschillende golflengten geen accumulatie van anthocyaan plaats (Hoofdstuk 2, 3, 4). Het feit dat jonge, in wit licht opgegroeide, *au* mutanten na 4 tot 5 dagen een geringe anthocyaansynthese vertonen, lijkt erop te wijzen dat fytochroom (of een B-fotoreceptor) na langere blootstelling van de *au* mutant aan licht functioneel wordt (Hoofdstuk 3). In bladeren van volwassen, in licht opgegroeide, planten van de *au* mutant is echter geen anthocyaan detecteerbaar (Hoofdstuk 5). Deze gegevens wijzen erop dat de capaciteit van de *au* mutant om anthocyaan te produceren ernstig is aangetast en dat het fytochroomtype dat in de *au* mutant ontbreekt hierbij een belangrijke rol speelt. Een ander door fytochroom gereguleerd proces, de remming van de lengtegroei van het hypocotyl, is ook aangetast in de *au* mutant in vergelijking met het wildtype. Behalve blauw licht (B) en UV-A heeft geen van de gebruikte golflengten remming van de hypocotyllengte tot gevolg, als zaailingen gedurende korte perioden worden blootgesteld aan licht van verschillende golflengten (Hoofdstuk 2, 3, 4). Dit impliceert dat het fytochroomtype dat in de *au* mutant ontbreekt een belangrijke rol speelt bij de regulering van de lengtegroei van het hypocotyl. Het feit dat B en UV-A als enige kleuren wel leiden tot remming van de lengtegroei suggereert dat er naast fytochroom een andere (B/UV-A) fotoreceptor werkzaam is. De internodia van volwassen, gedeëtioleerde *au* mutanten uit de kas zijn echter slechts een weinig langer dan die van het wildtype (Hoofdstuk 5). Bovendien hebben deze planten de capaciteit om op een eind-van-de-dag FR behandeling te reageren niet verloren (Hoofdstuk 5). De eind-van-de-dag FR behandeling is een methode om een schaduwmijdende reactie te induceren. Planten die zich onder een bladerendek van andere planten bevinden, staan bloot aan relatief weinig R en veel FR in vergelijking met planten in de volle zon, omdat bladeren R absorberen. Als strategie om het licht te bereiken, leidt dit tot extra lengtegroei van de planten die zich in de schaduw bevinden (schaduwmijdende reactie). Tot slot blijven *au* mutanten gedurende hun gehele leven geelgroen tengevolge van chlorofylgebrek (Hoofdstuk 5).

De resultaten van de experimenten die met de *lh* mutant van komkommer en het wildtype uitgevoerd werden, staan vermeld in hoofdstuk 6. De groeisnelheid van het hypocotyl van komkommerzaailingen van de *lh* mutant en het wildtype is hetzelfde in donker en continu FR. In continu R en B is de remming van de groei echter significant minder in de *lh* mutant, d.w.z. het hypocotyl van de *lh* mutant is onder deze omstandigheden langer dan dat van het wildtype. *In vivo* spectrofotometrische metingen laten zien dat geëtioleerde zaailingen van de *lh* mutant en het wildtype gelijke hoeveelheden fytochroom bevatten. Echter na een 4-daagse blootstelling aan continu FR, R of B bevat de *lh* mutant minder fytochroom dan het wildtype. Omdat de snelheid van afbraak en hersynthese van PI gelijk is in het wildtype en de mutant, wordt geconcludeerd dat de *lh* mutant het in licht stabiele fytochroom (PII) mist.

Om de taak van het in de mutant ontbrekende fytochroomtype te bepalen is, behalve kennis over de fysiologische responsen, kennis over de fytochroomstatus van de mutanten nodig. De fytochroomstatus van planten omschrijft welke fytochroomtypen aanwezig en functioneel zijn. Combinatie van gegevens

over de fytochroomstatus van de mutanten en hun wildtypen en de fysiologische kenmerken van de mutanten in vergelijking met hun wildtype maakt het mogelijk de taak van het fytochroomtype dat ontbreekt te bepalen. Hiertoe heb ik mijn eigen resultaten naast gegevens uit relevante literatuur gelegd (Hoofdstuk 7). Dit leidt tot de hypothese dat in de *au* mutant phy A ontbreekt, terwijl phy B aanwezig is op hetzelfde niveau als in het wildtype. In de *lh* mutant wordt de tegenovergestelde situatie gevonden. Behalve de *au* en *lh* mutanten worden ook andere mutanten besproken in hoofdstuk 7. Vergelijking van de mutanten met hun wildtypen toont aan dat in de *au* mutant andere processen worden aangetast dan in de *lh* mutant, wat de hypothese dat verschillende fytochroomtypen verschillende taken hebben, ondersteunt. Zo blijkt het phy B verantwoordelijk te zijn voor schaduwmijdende responsen. Bij andere in hoofdstuk 7 besproken processen: kieming, lengtegroei en anthocyaansynthese lijken beide fytochroomtypen (phy A en phy B) een rol te spelen. Het verschil tussen phy A en phy B voor wat betreft de stabiliteit in licht zou een indicatie kunnen zijn voor de rol die het speelt tijdens de verschillende ontwikkelingsstadia van de plant. De relatief grote hoeveelheid fytochroom tijdens de overgang van donker naar licht kan een mechanisme zijn om de gevoeligheid voor licht te vergroten op dit voor de plant cruciale moment. Gedurende deëtiolatie wordt phy A afgebroken tot een evenwichtsniveau is bereikt. Hoewel het voor de hand ligt dat na deëtiolatie de rol van phy A relatief minder belangrijk wordt, terwijl die van phy B meer op de voorgrond treedt, zijn er aanwijzingen dat phy A een rol blijft spelen in gedeëtioleerde planten (bv. in de chloroplast ontwikkeling).

Transductieketenmutanten. Als de verschillende fytochroomtypen verschillende functies hebben, kan verwacht worden dat er een verschil voor wat betreft hun transductieketen(s) optreedt. Hiertoe is het nodig de transductieketens te vergelijken. De fytochroom transductieketen(s) is (zijn) echter alles behalve duidelijk en een genetische benadering kan helpen de keten(s) te ontrafelen. In dit proefschrift wordt een potentiële fytochroom-transductieketenmutant, de high pigment *(hp)* van tomaat, gekarakteriseerd. De resultaten van deze karakterisering worden hieronder samengevat.

In tegenstelling tot het wildtype is er in de *hp* mutant al anthocyaan meetbaar na één enkele verzadigende R puls (Hoofdstuk 2). Na korte voorbelichtingsperioden is de door fytochroom gereguleerde anthocyaan-accumulatie in de *hp* mutant 5 tot 10 maal groter dan in het wildtype. (Hoofdstuk 2, 3, 4). In het bijzonder de relatief sterke respons in groen licht is opvallend, omdat groen licht nauwelijks door fytochroom wordt geabsorbeerd (Hoofdstuk 4). Hoewel de *au* mutant in vergelijking met het wildtype niet in staat is tot anthocyaanproduktie, vertoont de *au,hp* dubbelmutant toch een kleine (3% van de *hp* mutant) R/FR omkeerbare respons (Hoofdstuk 2). Deze data tonen aan dat de *hp* mutatie, in tegenstelling tot de *au* mutatie, tot een verhoogde anthocyaanproduktie in vergelijking met het wildtype leidt. Ook de lengtegroei van het hypocotyl blijkt sterker geremd te zijn in de *hp* mutant dan het wildtype (Hoofdstuk 3, 4). In tegenstelling tot het wildtype kan in de *hp*

mutant de B voorbelichting vervangen worden door een R voorbelichting om tot maximale anthocyaansynthese en remming van de groei te komen (Hoofdstuk 3). Volwassen, gedeëtioleerde *hp* mutanten reageren normaal (zoals het wildtype) op een eind-van-de-dag FR behandeling. Dit houdt in dat de lengtegroei toeneemt en de anthocyaanaccumulatie afneemt (Hoofdstuk 5). Experimenten die uitgevoerd werden om de oorzaak van het fenotype (uiterlijk) van de *hp* mutant te verklaren, tonen aan dat: (i) er in vergelijking met het wildtype geen sprake is van een hogere Pfr concentratie of een reductie van de afbraak-snelheid van fytochroom; (ii) de *hp* mutant niet hypergevoelig is voor Pfr: (iii) de kinetiek van de anthocyaanaccumulatie in continu R hetzelfde is in het wildtype en de *hp* mutant (Hoofdstuk 4). De ontsnapping aan R/FR omkeerbaarheid laat zien dat zowel in het wildtype als de *hp* mutant de anthocyaansynthese na verschillende voorbelichtingen beëindigd met een R puls nog 50% omkeerbaar is als er een donkerperiode van 5 uur tussen de R en FR puls zit (Hoofdstuk 4). Dit impliceert dat het Pfr dat hier een rol speelt relatief stabiel is.

Concluderend kan opgemerkt worden dat de *hp* mutant, ondanks een met het wildtype vergelijkbaar fytochroomgehalte en -systeem, versterkte fytochroom gereguleerde responsen vertoont. De *hp* mutant is dus geen fotoreceptormutant. In tegenstelling tot het wildtype is in de *hp* mutant geen samenwerking tussen de B-fotoreceptor en fytochroom nodig, om maximale anthocyaansynthese en remming van de hypocotylgroei te verkrijgen. Met andere woorden: de *hp* mutatie bootst de werking van B na. Een recessieve mutatie, zoals in de *hp* mutant, betekent meestal dat er verlies van een functie optreedt. Er wordt daarom verondersteld dat er een remmingsfactor wegvalt in de *hp* mutant. Het is bijvoorbeeld mogelijk dat de fytochroomwerking in geëtioleerde zaailingen geremd wordt door het *Hp* genprodukt. Zowel blootstelling aan B als de *hp* mutatie blijkt de effectiviteit van dit (remmende) genprodukt te reduceren. Deducerend kan gezegd worden dat de *hp* mutatie een amplificatiestap in de fytochroomtransductieketen aantast. Deze amplificatiestap zou dan alleen de phy A-werking aantasten, omdat: (i) de phy A-loze *au,hp* dubbelmutant sterk gereduceerde of geen anthocyaansynthese vertoont; (ii) overexpressie van het *phyA*-gen tot versterkte fytochroomresponsen leidt; (iii) aan phy B gerelateerde schaduwmijdende responsen normaal zijn in de *hp* mutant. Hoewel er enkele andere transductieketenmutanten geïsoleerd zijn (Hoofdstuk 7), is de ontrafeling van de fytochroomtransductieketen(s) nog ver weg. Verder onderzoek naar de mutanten die in dit proefschrift besproken zijn, selectie van nieuwe mutanten en het kloneren van de gemodificeerde genen kunnen een belangrijke rol spelen in de opheldering van de werking van de transductieketens.

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NAWOORD

Om een proefschrift te produceren zoals dat nu voor u ligt, is het nodig een groot aantal experimenten te bedenken en daadwerkelijk uit te voeren. Ik heb dit natuurlijk niet alleen gedaan en wil op deze plaats de mensen die een bijdrage hebben geleverd bedanken.

De personen die mij het meest geholpen hebben zijn Dick Kendrick en Mariëlle Schreuder.

Ik vermoed dat er zonder Dick heel wat minder van dit werk was terechtgekomen. Wat ik erg gewaardeerd heb, is dat je bij Dick kunt binnenkomen wanneer je wilt zonder dat er agenda's getrokken hoeven worden. Eenmaal binnen was het niet altijd even gemakkelijk weer aan je werk te gaan, vanwege de lange discussies die vaak ontstonden. Dit leverde echter ook nog al eens extra tijd op, omdat ik er op deze manier achter kwam dat iets in mijn proefopzet niet deugde. De meeste mensen moet het zo langzamerhand zijn opgevallen dat ik erg graag in andere labs werk, vooral als die zich in het buitenland bevinden. Dick is wat dat betreft een heel geschikte begeleider gebleken. Ik had bij wijze van spreken het voorstel om in een ander lab te gaan werken nog niet gedaan of het was al geregeld.

Mariëlle kwam in 1989 bij ons werken en heeft mij tot vlak voor de voltooiing van het proefschrift geholpen. Zonder haar zou het niet mogelijk zijn geweest gegevens te blijven produceren, terwijl ik zelf, met wisselend succes, in het buitenland aan het werk was. Mariëlle is medeauteur van de hoofdstukken 4 en 5 en is verantwoordelijk voor de uitwerking en layout van de meeste figuren. Ze heeft echter niet alleen goed werk geleverd, maar is ook een heel leuke collega geweest de afgelopen drie jaar.

Sebastiaan Verduin, student van de Hogeschool van Amsterdam, afdeling Laboratoriumonderwijs, heeft gedurende bijna een jaar geprobeerd de geheimen van de fotomorfogenese te ontsluieren. Zijn inzet was enorm en ik hoop dat hij geen spijt heeft van zijn, volgens mij goede, beslissing om door te studeren aan de Landbouwuniversiteit. De resultaten van een deel van zijn onderzoek zijn opgenomen in hoofdstuk 4 van dit proefschrift.

Voor mensen die niet uit Wageningen komen zal het misschien niet helemaal duidelijk zijn, maar de selectie van interessante mutanten en de naam Maarten Koornneef kunnen bijna altijd in één adem genoemd worden. Dus ook de voor mijn onderzoek gebruikte mutanten zijn afkomstig van de groep van Maarten Koornneef van de vakgroep erfelijkheidsleer.

De thresholdbox die beschreven staat in hoofdstuk 4 werd ontworpen en gebouwd door Willem Tonk. Voor Willem, ik noem hem wel eens de Panamarenko van Wageningen, was de relatief eenvoudige thresholdbox misschien niet zo'n grote uitdaging, maar de box heeft goed gefunctioneerd.

In 1990 hebben we gedurende een aantal maanden met volwassen tomatenplanten gewerkt. Deze werden in samenwerking met Leen Peterse en Henk Melissen gezaaid, verspeend en verzorgd in het fytotron, dat ondertussen

draaiende werd gehouden met de hulp van Jan van Kreel. Jan en Leen hebben Mariëlle en mij overigens vaak geholpen als apparaten niet deden wat ze zouden moeten doen. Het prettige was dat ze altijd meteen konden helpen op het moment dat het nodig was.

Voor de presentatie van het werk tijdens werkbesprekingen en congressen speelden Alex Haasdijk, Paul van Snippenberg en Siep Massalt een belangrijke rol, omdat zij de figuren, foto's, dia's en posters verzorgden.

Ik denk dat ik één van de laatste der Mohikanen ben voor wat betreft het aanschaffen van een computer: ik heb er namelijk geen. Dit was echter geen enkel probleem, omdat onze vakgroep goed voorzien is van computers, die onderhouden worden (werden) door Rienk Bouma (en Michael Hegeman) en Leo Herben. Verder waren voor de schrijfperiode van dit proefschrift de efficiënte besprekingen met Wim Vredenberg en Dick Kendrick en de controle van de tekst door Chris Kendrick van groot belang. De tekst is overigens geprint in het ultramoderne bureau van Kendrick & Co. te Bennekom.

De andere collega's van PFO hebben misschien niet direct bijgedragen aan dit proefschrift, maar ze zorgden wel voor een prettige werksfeer.

Mijn ouders wil ik graag bedanken voor de steun (ook financiële) die zij mij hebben gegeven. Mede daardoor was het mogelijk om de studie biologie in Groningen te voltooien en dit promotie-onderzoek aan te vangen.

Hoewel ik moet toegeven dat het in Wageningen helemaal niet zo vervelend was als ik in eerste instantie verwacht had, ben ik altijd met veel plezier naar Groningen afgereisd. Eén van de voordelen van het feit dat Cees in Groningen is blijven wonen, is dat ik dat weekeinde niet kon werken. Bovendien zag ik op deze manier ook eens andere mensen; in Wageningen lijkt iedereen wel iets met de Lanbouwuniversiteit te maken te hebben. Zonder Cees was dit proefschrift er vast ook wel gekomen, misschien zelfs dikker, maar wat was het leven dan saai geweest.

Na al deze mooie woorden moet ik toegeven dat het ook wel eens vervelend is zo diep op een onderwerp in te gaan als ik de afgelopen vier jaar gedaan heb. Zo kun je bijvoorbeeld nooit meer een mooie zin als: *'The bush is sitting under a tree and singing'*[1] lezen zonder direct te denken aan de hoeveelheid ver-rood licht waaraan deze 'bush' wel niet zal blootstaan.

Janny Peters,
April, 1992.

[1]Fragment van Ojibwa lied. In: The Portable North American Indian Reader. Edited by F.W. Turner III.

CURRICULUM VITAE

Janny Lammerdina Peters werd geboren op 6 december 1961 te Gorssel. Na het behalen van het VWO diploma aan het Baudartius College te Zutphen, begon zij in 1980 met de studie biologie aan de Rijksuniversiteit te Groningen. Hier behaalde zij in 1983 het kandidaatsexamen en in 1987 het doctoraalexamen met als hoofdvak plantenfysiologie en bijvakken bodembiologie en milieukunde. In het kader van het doctoraalonderzoek werd onderzoek verricht bij de interfacultaire vakgroep energie en milieukunde en de vakgroep plantenfysiologie van de Rijksuniversiteit te Groningen; de afdeling biologie van de bodem van het instituut voor bodemvruchtbaarheid te Haren en de afdeling botanie van de universiteit van Californië te Riverside (Verenigde Staten). Van 1 april 1988 tot 1 april 1992 was ze werkzaam als assistent in opleiding bij de vakgroep plantenfysiologisch onderzoek aan de Landbouwuniversiteit te Wageningen. Het onderzoek aan deze vakgroep heeft geleid tot dit proefschrift. Tijdens het promotie-onderzoek werd een periode aan de universiteit van Freiburg in Duitsland en het RIKEN instituut in Wako-shi te Japan doorgebracht. Vanaf september 1992 zal zij aan de universiteit van Californië te Santa Cruz onderzoek doen naar de rol van eiwit-RNA interacties bij de regulatie van de RNA stabiliteit, daarbij gebruik makend van rbcS mRNA van *Lemna gibba* als modelsysteem.