MUTANTS AS AN AID TO THE STUDY OF HIGHER PLANT PHOTOMORPHOGENESIS

MUTANTEN ALS HULPMIDDEL BIJ DE BESTUDERING VAN DE FOTOMORFOGENESE VAN HOGERE PLANTEN

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MUTANTS AS AN AID TO THE STUDY OF HIGHER PLANT PHOTOMORPHOGENESIS

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op vrijdag 11 november 1988
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

ISN 90 334

This work was supported by a grant from the Agricultural University Wageningen and performed at the Laboratory of Plant Physiological Research, Agricultural University, Generaal Foulkesweg 72, 6703 BW WAGENINGEN, The Netherlands.

STELLINGEN

1. De conclusie van Drumm-Herrel & Mohr en Mancinelli & Schwartz, dat één enkele lichtpuls geen invloed heeft op anthocyaan synthese in de hypocotyl van tomaat is onjuist.
   - Drumm-Herrel, H., and H. Mohr (1982). Photochem. Photobiol. 35, 233-236.
   - Mancinelli, A.L., and O.M. Schwartz (1984). Plant Cell Physiol. 25, 93-105.
   - dit proefschrift.

2. Het feit dat mutanten met een verminderde fytochroom concentratie lang worden tijdens groei in het donker bewijst dat de verrood licht absorberende vorm van fytochroom de fysiologisch aktieve vorm is van deze fotoreceptor.
   - dit proefschrift.

3. De grote hoeveelheid labiel fytochroom, aanwezig in geëtioleerde planten, speelt, in tegenstelling tot een tot nu toe gangbare opvatting (Hillman, 1972), wel degelijk een rol in de regulatie van zowel hypocotyl strekking als anthocyaan synthese.
   - Hillman, W.S. (1972). in: Phytochrome, eds: K. Mitrakos and W. Shropshire Jr., Acad. Press London, 574-584.
   - dit proefschrift.

4. Glastuinders moeten rekening houden met het lichtregime van de buren om ongewenste fotomorfogenetische effekten te vermijden.

5. Als de concentratie van een fluorescerende verbinding in cellen wordt bestudeerd met een 'fluorescence activated cell sorter', moet de totale fluorescentie van een cel worden gecorrigeerd voor het volume van die cel.

6. Optimale biotechnologische produktie van waardevolle verbindingen uit plantecellen vereist, naast de huidige 'trial and error' methodes voor opbrengstverhoging, meer onderzoek naar reaktieketens en moleculaire regulatiemechanismen van de processen op (sub-)cellulair nivo.

7. Bij het citeren uit wetenschappelijke publikaties moet de volledige redenatie worden vermeld en niet slechts een uit het verband gehaalde frase hieruit.

8. Tafeltennis tijdens de lunchpauze verhoogt de slagvaardigheid gedurende de rest van de dag.

9. De nederlandse versie 'Speller' van het tekstverwerkingsprogramma 'Wordperfect' zou zakken voor het eindexamen Nederlands wegens een onvoldoende woordenschat.

Stellingen behorende bij het proefschrift 'Mutants as an aid to the study of higher plant photomorphogenesis' van P. Adamse.

Wageningen, 11 november 1988

VOORWOORD

De bedoeling van een voorwoord is dat de auteur even persoonlijk het woord richt tot de lezers. Ik wil graag van die gelegenheid gebruik maken om iedereen te bedanken, die mij geholpen hebben bij het tot stand komen van dit proefschrift.

Allereerst wil ik mijn co-promotor dr. R.E. Kendrick bedanken. Dick, jouw voortdurende betrokkenheid bij mijn onderzoek en enthousiaste discussies over experimenten en resultaten hebben er toe geleid, dat ik met veel plezier heb kunnen werken aan een onderwerp waar ik in het begin maar weinig kaas van had gegeten, maar waar ik me nu zeer bij betrokken voel.

Mijn hele onderzoek draaide om mutanten en zo langzamerhand is het vanzelfsprekend dat dan de naam van dr. ir. M. Koornneef van de vakgroep Erfelijkheidsleer opduikt. Maarten, graag wil ik ook jou bedanken voor het enthousiasme en de deskundigheid waarmee je mij altijd bijstond.

Mijn promotor Prof. W.J. Vredenberg wil ik graag bedanken voor het kritisch volgen van mijn onderzoek en het kritisch beoordelen van het manuscript. Doordat gedurende enkele maanden praktisch elke donderdagmiddag enkele uren voor mij konden worden gereserveerd, kon ik mij prima aan de toch wel krap geplande deadline houden. Dit heb ik zeer gewaardeerd.

Veel dank ben ik ook verschuldigd aan Patricia Verhoeven-Jaspers. Patricia, als jij niet zoveel en zo secuur experimenten had zitten doen terwijl ik weer eens met m'n hoofd in de computer zat, had ik nu nooit zoveel resultaten kunnen presenteren. Ik heb het vooral gewaardeerd dat we bij de af en toe vervelende tijdschema's steeds alles soepel samen konden regelen.

Ondanks dat het nu lijkt alsof ik zelf geen experimenten heb gedaan, wil ik ook nog de studenten Margo Sprik en Johan Bakker, die hun resp. stage en doktoraal onderzoek onder mijn begeleiding wilden uitvoeren, bedanken voor hun belangrijke bijdrage aan mijn onderzoek.

Een inmiddels berucht geworden onderdeel van mijn promotie onderzoek is de groeimeter. Dankzij de inspanning van een hele groep mensen, binnen en buiten het lab, heb ik er toch nog experimenten mee uit kunnen voeren. Enkelen wil ik graag met name bedanken. Met veel inventiviteit en deskundigheid is de groeimeter voor het grootste deel ontworpen door Wim Tonk. Wim, ondanks veel tegenslagen bij en tijdens de ontwikkeling van de groeimeter zijn je inspanningen nu toch beloond: het apparaat werkt! Verder wil ik ook graag Rienk Bouma (die een belangrijk deel van de electronica-

ontwikkeling voor z'n rekening nam), Peter Aukes, Michel Hegeman, diverse 'electronica-stagaires', Jan van Kreel, Ad Looijen, en Ad Veenendaal en Rinus van Ginkel (van TFDL) bedanken voor al hun inspanningen om de groeimeter te bouwen en vervolgens ook aan de praat te houden.

Ik wil ook graag Alex Haasdijk, Paul van Snippenburg en Siep Massalt bedanken voor het verzorgen van de vele figuren, posters, foto's en dia's voor mijn artikelen, presentaties en dit proefschrift.

Verder wil ik graag al m'n andere collega's van het laboratorium voor plantenfysiologisch onderzoek bedanken. Dankzij jullie samenwerking en de prettige sfeer op het lab, zowel professioneel als sociaal en sportief, zal ik altijd met plezier aan m'n promotie-tijd terug denken.

Chris Kendrick wil ik ook graag bedanken voor het consciëntieuze doorlezen en corrigeren van al mijn manuscripten en voor het 'scheidsrechter' spelen bij meningsverschillen over het taalgebruik. Ik vrees dat ik wel eens wat moeilijk te overtuigen was op dat terrein.

Mijn nieuwe werkgever, het onderzoeksinstituut Ital, ben ik zeer erkentelijk voor de gelegenheid die me is gegeven dit proefschrift af te ronden. Mijn collega's op het Ital zou ik willen bedanken voor de morele steun tijdens het schrijven en voor de mogelijkheid die ze me gaven me eens flink af te reageren tijdens enerverende partijen tafeltennis.

Uiteindelijk, last but not least, wil ik graag mijn ouders bedanken, die me stimuleerden te gaan studeren, me daarin ook steeds steunden en die door hun voortdurende belangstelling ook hun bijdrage leverden aan het tot stand komen van dit proefschrift.

CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

A	absorbance
au	*aurea* tomato mutant
BAP	blue light-absorbing photoreceptor
BL	blue light
chl	chlorophyll
D	dark(ness)
EMS	ethylmethanesulfonate
FR	far-red light
GA	gibberellin
GL	green light
H	cycling rate of phytochrome photoconversion
HIR	high irradiance response
hp	high pigment tomato mutant
LFR	low fluence response
lh	long hypocotyl cucumber mutant
*l*P	labile phytochrome
P	phytochrome
PAR	photosynthetically active radiation
Pfr	far-red light absorbing form of phytochrome
Pr	red light absorbing form of phytochrome
RL	red light
*s*P	stable phytochrome
UV-A	320-400 nm UV
UV-B	280-320 nm UV
VLFR	very low fluence response
WL	white light
$\Delta\Delta$ A	difference in absorbance difference
ϵ_r	extinction coefficient of Pr
ϵ_{fr}	extinction coefficient of Pfr
Φ_r	quantum efficiency of Pr
Φ_{fr}	quantum efficiency of Pfr
φ	proportion of P as Pfr at photoequilibrium established by a given wavelength
φ_{BL}	Pfr/P at photoequilibrium established by BL
φ_{RL+FR}	Pfr/P at photoequilibrium established by RL + FR

ABSTRACT

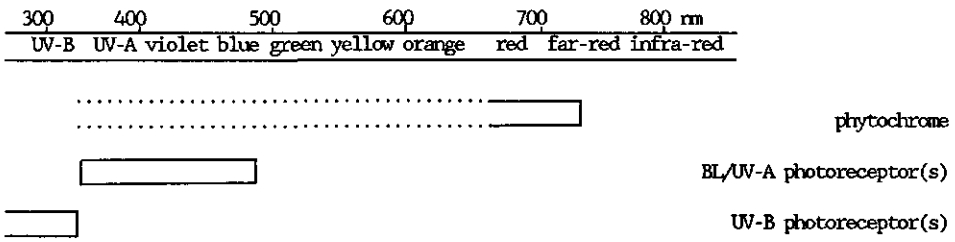
Study of photomorphogenesis is often complicated by the interaction of different photoreceptors regulating a given process or by the induction of multiple effects by a single photoreceptor. Mutants in which particular components of the morphogenetic pathways are eliminated provide the possibility of studying a more simplified form of photomorphogenesis. Three classes of photomorphogenetic mutants are proposed: photoreceptor, transduction chain and response mutants. In this study three mutants have been used: two have an elongated hypocotyl when grown in white light (the *aurea* (*au*) tomato mutant and the long hypocotyl (*lh*) cucumber mutant) and one with an enhanced pigment synthesis (the high pigment (*hp*) tomato mutant). The *au* mutant appears to be a photoreceptor mutant, lacking spectrophotometrically and immunochemically detectable labile phytochrome (*l*P). The *lh* mutant is proposed to lack stable phytochrome (*s*P) or its function. These mutants enabled the role of *s*P, *l*P and blue light (BL)/UV-photoreceptor(s) in several photophysiological processes to be studied. The results of these experiments indicate that *l*P plays a role in both hypocotyl elongation and anthocyanin synthesis in etiolated seedlings. This provides direct evidence that the 'bulk' *l*P is functional. In etiolated seedlings the *au* tomato mutant with its deficiency in *l*P is 'red-blind' and has a shift of fluence rate response curves for hypocotyl inhibition by BL and UV-A approximately 1 order of magnitude to higher fluence rates. In light-grown plants it is proposed that *s*P regulates the end-of-day far-red light (FR) response and the inhibition of hypocotyl elongation due to light perception by the cotyledons. Furthermore, these mutants with reduced phytochrome (P) content provide direct experimental evidence that the FR absorbing form of P (Pfr) is the active form indeed. If removal of the red light (RL) absorbing form of P (Pr) is the active photomorphogenetic process, instead of an increase of Pfr, seedlings with a reduced P content would be expected to be short. However, dark-grown seedlings of *lh* mutant and *au* mutant are both elongated. The *hp* mutant with its enhanced anthocyanin synthesis has enabled induction of anthocyanin synthesis in tomato seedlings in response to a single RL pulse to be observed, whereas in wild type this synthesis it too low to be measured. Study of anthocyanin synthesis with the aid of the *hp* mutant, the *au* mutant and the *au/hp* double mutant supports the conclusion that P is the terminal photoreceptor involved in tomato and that BL (operating through the BL/UV-photoreceptor or P) sensitizes the seedling to

P action at a later stage. Using a computer-controlled apparatus for continuous growth measurement, designed and constructed for this study, it has been possible to show the differences in kinetics of hypocotyl inhibition by BL or RL in both the *lh* mutant and its wild type. In BL inhibition started almost immediately after the onset of irradiation, whereas in RL a lag period of several hours was observed.

1. INTRODUCTION

1.1 The complexity of photomorphogenesis

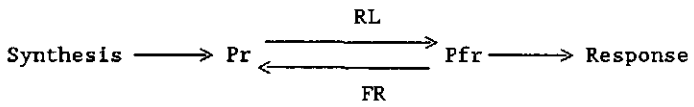
Plants not only depend upon light as an energy source (photosynthesis), but also utilize it as a source of information, enabling growth and development to be tuned to the prevailing light environment (photomorphogenesis). A number of photoreceptor pigments have evolved enabling information to be sensed over the complete daylight spectrum, since it is physically impossible for a single photoreceptor to fulfil this task with the accuracy required (Mohr, 1986). These include phytochrome, operating predominantly in the red (RL)/far-red (FR) spectral range, blue (BL)/UV-absorbing photoreceptor(s), operating in the BL spectral range and UV-B photoreceptor(s).



These pigments detect parameters of the light environment, such as light quality, quantity, direction and duration, and enable developmental responses to be controlled accordingly. Many processes in plants are controlled by these photoreceptor systems, such as, germination, elongation growth, apical hook opening, enzyme activity, leaf expansion, phototropism and flowering.

Phytochrome is the most extensively studied and characterized photomorphogenetic photoreceptor in higher plants. Its existence and photochromic properties were predicted by Borthwick and Hendricks on the basis of physiological experiments (Borthwick *et al.*, 1952). Phytochrome exists in two forms: Pr, which absorbs maximally in the RL spectral range at

around 660 nm; and Pfr, which absorbs maximally in the FR spectral range at around 730 nm. These two forms are reversibly interconvertible by light.

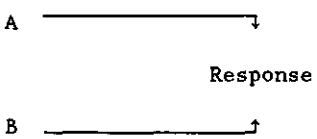


Recently the existence of multiple types of phytochrome (labile and stable phytochrome) has been suggested (Vierstra and Quail, 1986; Chapter 4). In addition, there appear to be multiple working mechanisms of phytochrome: very low fluence response (VLFR), low fluence response (LFR) and high irradiance response (HIR) (Chapter 3).

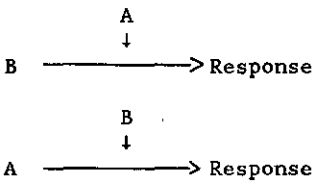
The BL/UV-photoreceptor(s) is/are hypothetical, being postulated on the basis of action spectra. As early as 1864 Julius Sachs demonstrated that phototropism is only effected by the BL region of the spectrum. Subsequently a wide range of physiological BL/UV effects have been studied (see Senger and Schmidt, 1986 for review). The predicted photoreceptor which corresponds to many action spectra having maxima or shoulders at (370), 420, 450 and 480 nm (Senger and Lipson, 1987) has been called cryptochrome (Gressel, 1979; Schäfer, 1982; Senger and Schmidt, 1986) or BAP (= BL-Absorbing Photoreceptor) (Thomas, 1981), but BL- and/or UV-A-receptor is used by many workers. On the basis of action spectra it has been predicted that the BL/UV-A photoreceptor is a flavin or a carotenoid, but no final proof is yet available (Senger and Schmidt, 1986; Horwitz and Gressel, 1986). However, flavins rather than carotenoids are strongly suggested to be the photoreceptor pigments for BL/UV-A (Mohr *et al.*, 1984; Drumm-Herrel and Mohr, 1982a; Drumm-Herrel and Mohr, 1982b). The nature of the UV-B absorbing pigment(s) has yet to be elucidated.

The different photoreceptors can have several modes of (co)-action (e.g. independent or interdependent) (Mohr, 1987). With independent co-action both photoreceptors can elicit the same response, while with interdependent co-action there are several possibilities. Schematic it can be visualized as in the figure below, with A and B representing two hypothetical photoreceptors.

Independent:



Interdependent:



The mode of co-action and the relative importance of the different photoreceptors is still unclear; some species appear to respond only to phytochrome, whereas others utilize both phytochrome and BL/UV-A-photoreceptor(s) (Gaba and Black, 1979; Holmes, 1983; Attridge *et al.*, 1984; Morgan *et al.*, 1980; Chapter 3).

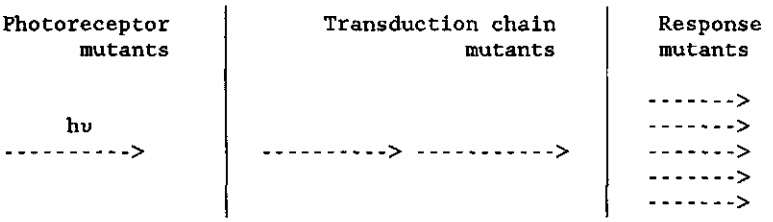
The existence of different photoreceptors, multiple photoreceptor types and working mechanisms means that photomorphogenesis is rather complex. The presence of photoreceptors, having overlapping absorption spectra, influencing the same response, makes it very difficult to find answers to several long standing questions concerning photomorphogenesis. It is especially difficult to distinguish the photoreceptors which play a role in a particular response; due to maintenance of at least some active Pfr by light throughout the daylight spectrum it is impossible to prove that Pfr is not required for a given response (Gaba and Black, 1987). It is also difficult to discriminate between responses controlled by multiple types of a photoreceptor. For example, where the labile and stable types of phytochrome are both present action spectra would be indistinguishable for a particular physiological response (Pratt and Cordonnier, 1987).

1.2 The use of photomorphogenetic mutants

The availability of mutants, in which certain parts of the morphogenic pathway are eliminated, provides a useful tool for the study of photomorphogenesis. Such genotypes exhibit a simplified photomorphogenesis.

The relevance of the deletion in the mutant is directly indicated by the difference between the mutant and its isogenic wild type. Such mutants could provide the potential to ascribe a response to a particular phytochrome type and, where photoreceptor co-action has been established, they will enable the relative importance of different photoreceptors to be established.

Mutants that lack a particular photomorphogenetic response may be defective in the photoreceptor, in the transduction chain or in the responses itself (Koornneef and Kendrick, 1986). Photomorphogenetic mutants can therefor be divided simplistically into three groups:



Photoreceptor and transduction chain mutants would be pleiotropic for all responses under control of a particular photoreceptor whereas a response mutant would be restricted to modification of one particular response. Only a few photomorphogenetic mutants have been identified (Chapter 2). The present study utilizes examples of different mutants, assigned to one or other of these catagories, to study photomorphogenesis.

1.3 Outline of the present study

Photomorphogenetic mutants of tomato and cucumber have been used in this study, which exhibit reduced photo-inhibition of hypocotyl growth in white light, compared to their isogenic wild types. Initially a detailed characterization of the mutants was carried out. Subsequently experiments were designed in an attempt to unravel a number of photomorphogenic problems.

Chapter 2 summarizes literature on photomorphogenetic mutants of higher plants and outlines the initial characterization of the mutants used in this study.

Chapter 3 reviews of the literature on the photophysiology of seeds and seedlings of cucumber and tomato. Current theories are discussed in relationship to the photomorphogenesis of cucumber and tomato.

Chapter 4 describes the spectrophotometric measurement of the phytochrome contents of seeds, dark-grown seedlings and light-grown plants of both cucumber and tomato. Phytochrome measurements using immunochemical techniques are also described. These latter experiments were carried out in collaboration with Prof. M. Furuya and coworkers in Japan (cucumber) and Prof. P.H. Quail and coworkers in the USA (tomato).

Chapter 5 describes the results of long-term physiological experiments with cucumber.

Chapter 6 describes the results of short-term growth experiments with cucumber, together with a detailed description of equipment developed for computer-controlled, continuous growth measurement.

Chapter 7 describes the results of long-term physiological experiments with tomato.

Chapter 8 describes the results of experiments on anthocyanin synthesis in tomato seedlings. In these experiments a mutant which exhibits an enhanced anthocyanin response to light compared to the wild type has been used.

Chapter 9 discusses conclusions and hypotheses proposed in the previous chapters and prospects for the use of the mutants in photomorphogenetic studies.

Parts of the work presented in this thesis have been published or are accepted for publication (Adamse *et al.*, 1987; Parks *et al.*, 1987; Koornneef *et al.*, 1987; Adamse *et al.*, 1988a; Adamse *et al.*, 1988b (in press); Adamse *et al.*, 1988c (in press)) or are in preparation for publication.

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2. PHOTOMORPHOGENETIC MUTANTS

2.1 Introduction

Genotypes (often as induced mutants) in which certain parts of the photomorphogenetic pathway are eliminated, provide important tools for physiological analysis. Such genotypes will exhibit a photomorphogenetic pattern different from and often simpler than that of their wild type. The relevance of the deleted part in the genome of the mutant is directly indicated by its difference in response compared to its isogenic wild type. Mutants with other defects, such as chlorophyll (Chl) or carotenoid deficiency, can also be very useful in photomorphogenetic research.

2.2 Sources of genetic variation

Genetic variation for photomorphogenetic responses can be obtained in a number of ways. Some occurs spontaneously and appears as natural variation within a plant species. However, in general variation is achieved most effectively by artificial treatments.

Mutagenesis is normally induced either by chemical treatment or irradiation with ionizing radiations. Most induced mutations are recessive: a gene loses its function upon mutation, but the corresponding wild type allele provides sufficient gene product to 'mask' the mutation. For homozygous diploid plant species recessive mutants can be detected in the progeny of mutagen treated plants. This progeny, called the M_2 generation, is obtained by selfing the plants that now are heterozygous due to the mutagen treatment. A complication with mutagen treatment is the occurrence of more than one mutated gene, especially after the application of high mutagen doses. In this situation backcrossing of the mutant to its wild type is required to eliminate these disturbances in the genetic background. Although mutations most often occur in the nuclear genome resulting in mutants that show Mendelian segregation ratios, they may also affect organellar DNA. Some mutations in the chloroplast DNA result in defective plastids and such defects show maternal inheritance.

It has been recently recognized that tissue culture, through a variety of molecular mechanisms, often acts as an additional effective means of mutagenesis. Passage of homozygous plants through a stage of tissue culture

may result in monogenic variants. In general these are initially detected in the progeny of the tissue culture derived plants. This type of genetic variation is called somaclonal variation (Larkin and Scowcroft, 1981; Lee and Phillips, 1988).

As a result of artificial or natural selection, genetic variants are available which can be exploited for the study of specific physiological responses. This genetic variation can be related to physiological adaptation e.g. in the case of differences in the sensitivity to daylength. However, it is also possible that differences in photomorphogenic responses hardly affect plant phenotype under normal cultivation conditions. Such differences will be detected only in specifically designed physiological experiments. Examples of these are genetic variations in the relative contributions of blue (BL) and red (RL) light in anthocyanin formation in maize (Beggs and Wellmann, 1985) and cabbage (Sponga *et al.*, 1986) and in the presence or absence of recovery of growth rate after irradiation with a short pulse of BL in cucumber (Cosgrove, 1981). To draw valid conclusions about the physiological mechanisms underlying these differences it will be necessary to know the exact genetic basis. This has not been investigated in most cases. The extent of the variation found among wild and cultivated species is in practice somewhat limited, because it will not include extreme variants with a strongly reduced chance of survival (in nature) or with reduced yield (for cultivated species).

2.3 Types of photomorphogenic mutants

2.3.1 Phytochrome mutants

As described in Chapter 1, photomorphogenetic mutants can be divided simplistically into three groups: photoreceptor-, transduction chain- and response mutants.

2.3.1.1 *Photoreceptor mutants* Spectrophotometric and immunological assays are available for the detection of phytochrome (P), therefore it is relatively easy to test for the presence of the bulk light-labile pool of the photoreceptor. However, the existence of multiple photoreceptor types and the presence of several structural genes for P synthesis of the individual P types makes the system more complex. Perhaps in practice it is only possible to obtain leaky mutants. Nevertheless, such mutants enable the

concentration of the photoreceptor to be modified without irradiation. In such systems it will be possible to carry out valuable physiological experiments which would otherwise be impossible.

A mutant lacking P would enable the importance of cryptochrome to be assessed and help to understand the mode of co-action of cryptochrome and P, since both absorb BL. This would be particulary useful in the case of prolonged irradiation responses. A mutant lacking one of the P types could help understand the importance of light-labile (*lP*) and light-stable P (*sP*) in different responses.

The long-hypocotyl mutants at the *hy-1* and *hy-2* loci of *Arabidopsis* were the first recognized photoreceptor mutants of higher plants (Koornneef *et al.*, 1980). These mutants have been isolated on the basis of their increased hypocotyl growth, relative to wild type, when grown in white light (WL). Both of these mutants lack spectrophotometrically detectable P in seeds and etiolated seedlings.

A study of the photocontrol of seed germination of these mutants (Cone, 1985; Cone and Kendrick, 1986) revealed that the germination remained under P control despite the apparent lack of the photoreceptor as indicated by spectrophotometry. However, in most cases sensitivity towards RL was reduced when compared to wild type. It was speculated that the P involved in the control of seed germination was not the bulk labile pool, which is involved in the inhibition of hypocotyl growth during de-etiolation. Light-stable P is a possible candidate for the photo-induction of seed germination and is probably synthesized within the seed during maturation on the mother plant. The effect of the light conditions during the latter stages of seed development on the subsequent dark-germination and sensitivity to RL, was shown by Gettens-Hayes and Klein (1974) for *Arabidopsis*.

2.3.1.2 *Transduction chain mutants* Mutants that contain a particular photoreceptor, but lack all photomorphogenetic responses associated with it, are most probably transduction chain mutants. Possible mutants which fit these criteria are those at the *hy-5* locus in *Arabidopsis* (Koornneef *et al.*, 1980). Approaches have been suggested which use mutant selection in specific transgenic plants. These aim specifically at the isolation of mutations in trans-acting factors that interact with the promotors of light regulated genes (Schäfer, 1987; Karlin-Neumann and Tobin, 1987; Chory and Ausubel, 1987). If these trans-acting factors are extremely gene specific the mutants isolated with these protocols will be response mutants.

Mutations may not only lead to the absence of a particular photoreceptor or response but may also result in an amplified response. Such high-response mutants would facilitate the study of a photoresponse. It has been proposed that in the regulation of many processes in plants the differences between cells at different stages of development are entirely quantitative. The reactions involved are therefore proposed to be already occurring in darkness (D) albeit at low rates and in the case of light-activation only the rate of the process is enhanced (Mohr and Schäfer, 1983). Whether this enhancement can take place in a cell depends upon its 'competence'. Competence of a cell means that the cell is able to respond to a specific stimulus with a specific response. In the case of photomorphogenesis, P can only exert its control function once a particular cell or tissue has reached competence (Mohr and Schäfer, 1983). This causes differences in P control of a response in cells at different stages of development. If this is true, then the levels of activity in D may be below detection limits when cells have not yet reached competence. A high-response mutant with its amplified response could provide the level of activity to support this hypothesis.

Anthocyanin synthesis, a process which is strongly light regulated and under control of at least three photoreceptor pigments (Beggs *et al.*, 1986), provides a system to illustrate the usefulness of such mutants. While many of the anthocyanin deficient mutants are response mutants, a number are particularly interesting since in many species it appears that P plays a very important role. Phytochrome mutants would therefore be expected to lack photocontrol of anthocyanin synthesis, but in addition would be pleiotropic for other effects.

2.3.1.3 *Response mutants* By definition, a response mutant will be a mutant modified with respect to a specific response. Mutations in structural parts, as well as in regulatory parts of light-inducible genes, fall into this category. The latter group may lead to a constitutive expression of the gene in question. Especially when the latter steps in the transduction chain are gene specific, mutations in these factors also result in mutants that will be classified as response mutants. Examples are mutants concerning photoperiod and day-length sensitivity and flowering. As well as differences between species in relationship to photoperiodic induction of flowering, variation within species for daylength sensitivity has frequently been reported (Murfet, 1977). Probably with the exception of the grass *Themeda*

australis, where short-day (SD), long-day (LD) and daylength-neutral (DN) ecotypes have been identified, variation within a species generally ranges from daylength-sensitive to daylength-insensitive (SD or LD to ND). Genetic differences for this character are often found to be based only on a limited number of genes. Although the exact physiological nature of these genes is not known, they seem to affect only the flowering response and no other photomorphogenic responses except those related to flowering. Therefore these genes should be classified as response affecting genes.

2.3.2 Blue-light/UV-A photoreceptor (cryptochrome) mutants

While considerable progress has been made with the study of the photoreceptors absorbing in the BL region of the spectrum with lower organisms such as *Phycomyces* (Lipson, 1987), studies with higher plants have been surprisingly limited. The long hypocotyl *Arabidopsis* mutants (*hy-4*) (Koornneef *et al.*, 1980), appear to be defective with respect to BL-absorbing pigment(s). Such mutants provide a direct indication that a photoreceptor other than P, absorbing in the BL region of the spectrum, plays a role in the regulation of growth, since they are modified independently in the BL region, while response in the RL and FR spectral region is retained.

One of the long standing problems in the photobiology of higher plants is an understanding of phototropism. At face value, selection of phototropic mutants is simple. Recently Poff and co-workers have isolated phototropic *Arabidopsis* mutants (Poff *et al.*, 1987; Khurana and Poff, 1988), some of which in addition to reduced phototropism showed no geotropism. This is a situation similar to *Phycomyces*, where some mutants are defective in all curvature responses, whereas others were specifically affected in phototropism. Only the latter were considered to be true photoreceptor mutants (Lipson, 1987). It will be interesting to see if such phototropic mutants also lack the specific BL effect on straight growth (Drumm-Herrel and Mohr, 1985; Cosgrove, 1986).

Comparing two albino mutants of *Helianthus* which have reduced Chl and carotenoid contents with their wild type, Wallace and Habermann (1959) observed normal phototropism. This indicates that the photoreceptor involved in phototropism is not a carotenoid.

2.3.3 UV-B photoreceptor mutants

While little work has been carried out with mutants in relationship to

BL/UV-A-absorbing photoreceptors, even less is known about photoreceptors absorbing in the UV-B region of the spectrum. The problems of interpretation of long-term experiments are very similar to those in the BL/UV-A spectral region, since UV-B absorbed by the protein and/or chromophore of P produces significant amounts of Pfr under continuous irradiation conditions. This fact makes the study of photoreceptor co-action difficult. While ingenious experiments have clearly implicated a separate UV-B photoreceptor in a number of species (Wellmann, 1971; 1976; Drumm-Herrel and Mohr, 1981) the relative role played by P and UV-B photoreceptors remains largely unknown. For example, wheat seedlings (*Triticum aestivum*, cv. Schirokko) can only produce anthocyanin after a pretreatment with UV-B, whereas other wavelengths are ineffective (Mohr and Drumm-Herrel, 1983). However, following such a pretreatment P has been shown to play an important role. An analysis of different cultivars of maize has demonstrated the existence of genetic variability with respect to anthocyanin synthesis in response to UV-B (Beggs and Wellmann, 1985). This demonstrates that suitable genotypes to facilitate study may be found among either cultivars or biotypes in the case of natural populations.

2.3.4 Other mutants useful in photomorphogenetic research

Mutants lacking photosynthetic pigments have played an important role in photosynthesis research (Somerville, 1986). They can also be useful in the study of photomorphogenesis. For example, it is possible to test for the involvement of other photosystems in the absence of photosynthesis. Mutants deficient in photosynthetic pigments enable the role of other photoreceptors in a particular physiological response to be observed. An example is the relative roles of light absorbed by photosynthetic pigments and other photoreceptors in the photocontrol of stomatal movement (Zeiger, 1986). Isolated achlorophyllous stomata of the orchid *Paphiopedilum* opened in response to BL but not to RL, while stomata in intact leaves, surrounded by chlorophyllous tissue, showed a response to both BL and RL. This indicates that besides photosynthesis, an additional photo-process is involved in this response. Experiments carried out by Habermann (1973; 1974) with albino mutants of *Helianthus* indicated that photosynthesis is not involved in the light-dependent stomatal opening. These experiments suggested that P was also involved, together with a specific BL-dependent photoreaction.

A complete lack of photosynthetic pigments would obviously be lethal. Genes for albinoism can be maintained in 'cultivation' either as

heterozygotes or as seedlings studied before the stored food reserves, deposited in the seed while on the mother plant, are exhausted (Somerville, 1986). Such albino mutants seedlings of barley have been used by Borthwick *et al.*, 1951), before P had been identified, to demonstrate that photosynthetic pigments were not involved with the photomorphogenetic processes associated with de-etiolation. Alternatively some albino phenotypes have been studied after grafting them onto normal green plants (Wallace and Habermann, 1959). Albino seedlings also provide a suitable tissue for the spectrophotometric measurement of P in de-etiolated seedlings. White regions of variegated leaves have also been used to demonstrate the existence of P in light-grown plants of a number of species (Spruit, 1970).

Screening as a consequence of high Chl content can effect light-regulated processes in plants. Herbicides, such as Norflurazon, which result in bleached seedlings have been used in photomorphogenesis research to prevent the artifacts. However, comparing the response of two cultivars of cabbage differing in Chl content with such bleached seedlings it appears that the use of bleaching agents can produce undesirable side effects (Mancinelli *et al.*, 1988). The use of genotypes with reduced Chl content could be a safer approach. Recently Piening and Poff (1988) have used carotenoid deficient mutants of maize (Robertson, 1975) to demonstrate the importance of screening in establishing the light gradient within the coleoptile necessary for light direction detection in phototropism.

Hormone mutants provide a means of testing the involvement of hormones in the transduction chain between perception by the photoreceptor and the ultimate physiological response. An example is the hypothesis that gibberellin (GA) is an integral part of the P transduction chain that results in the promotion of seed germination (Hilhorst and Karssen, 1988). The effect of P may either mimic GA action or influence the tissue sensitivity towards endogenous GA's. Furthermore, the P-controlled end-of day far-red (FR) response has been proposed to be a function of the level of GA_1 since pea mutants that appeared to be blocked at this step, show a much reduced response (Jolly *et al.*, 1987). Alternatively, the involvement of P in GA synthesis has been shown in the *le* pea mutant, where a Pfr-induced blockage of 3β-hydroxylation of GA_{20} to GA_1 causes a decrease in elongation growth of light-grown plants (Campell and Bonner, 1986; Sponsel, 1986). The use of such mutants clearly offers a more elegant way of controlling internal hormone levels than the application of inhibitors which always have

problems of uptake, transport and specificity of action associated with their use.

Modification of GA metabolism either by inducing an over-producion of GA or an increase in sensitivity to GA, may give rise to tall growing (giant) plants. The *lv* pea mutant has a similar biosynthesis and degradation of GA as wild type, but appears specifically to have an enhanced sensitivity to GA_1 in the light (Reid and Ross, 1988). No difference is seen in D, therefore these workers concluded it is a photomorphogenic mutant. J.B. Reid (Pers. comm.) claims a large effect in RL, but not in FR.

The *pro* mutant of tomato (Stubbe, 1957) is another mutant with long internodes in WL, which is similar in leaf morphology to a wild type plant treated with GA. However, the data published argue against it being a GA over-producer (Jones, 1987; Jupe *et al.*, 1988). These giant mutants, which clearly resemble the wild type plants that have been grown under condition of supplementary FR to lower the φ value, are an additional source of potential photoreceptor mutants.

2.4 The isolation and preliminary characterization of three mutants used in this study

2.4.1 Tomato *au* mutant

During selection of GA mutants, a mutant was isolated, which had been induced by treating seeds of cv. Moneymaker with ethylmethanesulfonate (EMS) and required GA for germination. In contrast to GA-deficient mutants this mutant was characterized by a long hypocotyl and a marked reduction in Chl content when grown in WL (Koornneef *et al.*, 1981). The P level in etiolated seedlings of this mutant appeared to be reduced. A genetic analysis revealed that this mutant was allelic with the *aurea* (*au*) locus located on chromosome 1.

Mutants with a phenotype similar to that of *aurea*, although less extreme, have been called *yellow green* (*yg*). Another mutant allele was later isolated at the *yg-2* locus (called auroid) and has been mapped to chromosome 12 (Koornneef *et al.*, 1985). A third locus, *yg-6*, for which an *aurea* type mutant was described, has been demonstrated to be an *au* allele. The location of *yg-6* on chromosome 11 can be explained by a translocation between chromosome 1 and 11 during the irradiation induction of the *yg-6* mutant (Koornneef *et al.*, 1986). Another mutant at the *au* locus, also with

physiological characteristics similar to those of the previously described *au* mutants, was isolated recently in the progeny of tomato plants derived from tissue culture by Lipucci di Paola *et al.* (in press).

Perez *et al.* (1974) have suggested that the *yg-6* mutant phenotype could be explained by an over-production of GA's. However, the reduced seed germination of this and other *au* mutants is an unlikely phenotype for a GA over-producer (Koornneef *et al.*, 1985). Buurmeijer (1987) has examined photosynthesis in *yg-2* and has observed a reduced Chl *a/b* ratio compatible with previous observations of the smaller photosynthetic unit in *yg-6* (Miles, 1971). Presumably, this is an expression of the reduced P action via gene expression on chloroplast development. That Chl and chloroplast defects are associated with possible P photoreceptor mutations is also shown by the *hy-1* and *hy-2 Arabidopsis* mutants. Furthermore, reduced levels of P (40% of the level in the wild type) have been measured in a number of barley mutants deficient in Chl (Atkinson *et* al., 1980). However, most Chl mutations found in higher plants probably represent mutations at later stages in the Chl synthesis pathway. It must not be forgotten that P and Chl possess a common tetrapyrrole chromophore and therefore mutations in tetrapyrrole synthesis could lead simultaneously to deficiency in Chl and P.

2.4.2 Cucumber *lh* mutant

A mutant of cucumber was isolated in the progeny of irradiated *Cucumis* plants of complex hybrid origin (Van der Knaap and de Ruiter, 1978) which had very long hypocotyls when grown in WL. The leaves were lighter green than those of wild type, but not as different from wild type as the *au* tomato mutant. This monogenic recessive cucumber mutant was found to be allelic to a similar mutant (Koornneef and van der Knaap, 1983) described and named *lh* by Robinson and Shail (1981). The original mutant was crossed with the cucumber cultivar Stereo (Deruiterzonen Ltd.) and the subsequent generations were obtained by selfing. In the experiments described in this thesis F_6, F_7 and F_8 lines derived from this cross were used providing a strong genetic homogeneity within the lines. Mutant (*lh/lh*) and wild type (*Lh/Lh*) lines are derived from the same F_4 plant, thus a reasonable amount of isogenicity is present between them. This is also indicated by the similarity between wild type and mutant in characters not associated with the *lh* gene (e.g. ratio of female and male flowers; fruit characteristics). Seeds were obtained by self-pollinating plants growing in a greenhouse during the summer.

2.4.3 Tomato *hp* mutant

A mutant of tomato has been described by Reynard (1956), which has an exceptionally high pigment content, resulting in dark coloured stems and fruits. Subsequently the gene for this high pigmentation, *hp*, has been used by many breeders in an attempt to develop new varieties. It was noticed that in varieties containing this gene the level of anthocyanin synthesis was increased (Kerr, 1965; Ibrahim *et al.*, 1968). Furthermore, germination was retarded, seedling growth less vigorous than that of wild type (Thompson *et al.*, 1962; Kerr, 1960; 1965) and the carotenoid and ascorbic acid content of fruits was increased (Ibrahim *et al.*, 1968). The *hp* mutant also shows pleiotropic effects that may be associated with P, such as a reduced plant height especially under red and yellow light (Mochizuki and Kamimura, 1985) and higher Chl levels particularly in fruit tissue, both characteristics being opposite to the *au* phenotype. A mutant with enhanced anthocyanin synthesis could be very useful in the study of P control of anthocyanin.

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3. PHOTOPHYSIOLOGY OF SEEDS AND SEEDLINGS OF CUCUMBER AND TOMATO

Cucumber (*Cucumis sativus* L.) and tomato (*Lycopersicon esculentum* L.) are known to respond to both light absorption by phytochrome (P) and blue (BL)/UV-A-receptor(s) (Thomas and Dickinson, 1979; Cosgrove, 1981; Drumm-Herrel and Mohr, 1982b; Gaba and Black, 1979). Numerous processes, associated with growth and development of cucumber and tomato, have been studied. Describing and comparing these different light responses gives an insight into the photoregulation of growth and differentiation. In addition the possible co-action of different photoreceptors in these species is discussed. Responses of other species are discussed where relevant.

3.1 Hypocotyl elongation

3.1.1 Short-term irradiation

Brief (5 min) irradiation of plants with red light (RL), establishing a high Pfr/P ratio (φ) [where P is the total phytochrome (Pr + Pfr) and Pr and Pfr are the RL- and far-red light (FR)-absorbing forms respectively)], or BL, establishing an intermediate φ value, inhibits hypocotyl elongation in both etiolated and de-etiolated seedlings of cucumber. This effect can be reversed when immediately followed by a brief (10 min) irradiation with FR, establishing a low φ value (Meyer, 1968; Black and Shuttleworth, 1974; Cosgrove, 1981; Gaba and Black, 1985b).

Brief irradiation with FR at the end of a daily photoperiod (end-of-day treatment) stimulates the elongation rate of light-grown (de-etiolated) cucumber seedlings; this response can be reversed by RL (Gaba and Black, 1985a; 1987). A similar response can be elicited by BL; the extent of this end-of-day BL effect is similar to the stimulation of hypocotyl growth produced by a RL+FR mixture establishing the same φ value as established by BL (Gaba and Black, 1987). This indicates that the φ value established at the end of the light period to a great extent determines the elongation rate in subsequent darkness (D).

Apart from similarity between the responses to short-term irradiation with RL or BL there is also a clear difference: the time course of the growth responses in RL and BL differs in many species. Detailed growth measurements with etiolated cucumber seedlings demonstrate that BL-inhibition begins within 60 s and a fast recovery occurs in subsequent D,

whereas the RL-inhibition has a lag period of about 30 min and a slow recovery in D (Meyer, 1968; Cosgrove, 1981; Cosgrove and Green, 1981). Other species showed similar differences in lag periods after irradiation with BL or RL: *Sinapis* (Cosgrove, 1982), pea (Cosgrove, 1981).

De-etiolated cucumber seedlings show the same differences: rapid decrease of growth rate after 5 min exposure to BL and a fast recovery in D, whereas the RL-induced inhibition starts only after an extended lag period of 5-6 h and recovers in D with a lag period of about 8 h (Gaba and Black, 1979; Gaba and Black, 1983). However, shorter lag periods have been reported for de-etiolated seedlings of several other species. Morgan and Smith (1978) observed a rapid increase in stem extension rate of de-etiolated *Chenopodium album* seedlings induced by treatment of the whole plant with supplementary FR added to background white light (WL) (lag period of 7 min). This was also seen by Morgan *et al.* (1980) in similar experiments with de-etiolated *Sinapis* seedlings (lag period of 10-15 min). Apparently changes in P (Ú) during the main light period can manipulate growth rate within minutes in light-grown plants. The differences between these results and those of Gaba and Black may be a consequence of the different light treatments, species, tissue age, and pretreatment conditions used.

Experiments with *Sinapis* seedlings have clearly shown that supplementary FR had no effect on the growth inhibition by BL during the first 5 min of the response (Cosgrove, 1982) and it was concluded that this rapid BL inhibition was mediated by a specific BL-photoreceptor. The slower response with a lag period of hours was proposed as being mediated by P.

The complex response of de-etiolated cucumber seedlings to short-term irradiation with WL can be explained by assuming the involvement of two different photoreceptors. After onset of the WL an immediate decrease in hypocotyl growth rate was observed. After the 2-3 h irradiation period recovery in D was rapid, but 2 h later a second decrease in growth rate occurred followed by a slow recovery (re-etiolation) (Gaba and Black, 1979). It is likely that the first decrease and the first recovery are mediated by the BL-photoreceptor, whereas the second decrease and the second recovery are mediated by P.

Growth inhibition of tomato seedlings by short-term irradiation has hardly been investigated; only responses to end-of-day FR pulses have been reported. Similar to results with cucumber seedlings brief irradiation with FR at the end of the daily photoperiod stimulated the elongation rate of light-grown tomato seedlings (Tucker, 1975; Selman and Ahmed, 1962; Decoteau

et al., 1988).

3.1.2 Long-term irradiation

Continuous irradiation with RL or BL inhibits hypocotyl elongation compared to plants in D for both etiolated and de-etiolated seedlings of cucumber and tomato (Meyer, 1958a; Meyer, 1958b; Meyer, 1968; Black and Shuttleworth, 1974; Thomas and Dickinson, 1979; Koornneef *et al.*, 1985). However, irradiation with FR has different effects in etiolated and de-etiolated seedlings. Etiolated cucumber seedlings are inhibited by prolonged FR, however the effect becomes less apparent in older seedlings. De-etiolation appears to diminish the sensitivity to FR (Black and Shuttleworth, 1974). This phenomenon has also been observed in tomato seedlings (Thomas and Dickinson, 1979).

The interpretation of such high irradiance responses (HIR's) is clearly not simple. This is especially true for the observation that continuous irradiation with FR is more effective in inhibiting hypocotyl elongation of dark-grown seedlings of both cucumber and tomato than continuous irradiation with RL (Thomas and Dickinson, 1979). Nevertheless, the dual-wavelength experiments of Hartmann (1966) demonstrated that P can be implicated as the photoreceptor responsible for the sharp response effectiveness at 716 nm in the case of lettuce. The FR maximum has been explained on the basis of the Ú ratio which results in the maintenance of the highest amount of Pfr over the experimental time period. It reflects a compromise between two competing reactions: one reaction in which Pfr inhibits growth, and a second in which the amount of P is reduced due to destruction of Pfr. The most effective light should be that which establishes a level of Pfr low enough to minimize the rate of Pfr destruction, yet sufficient to inhibit growth. While RL maintains a higher φ value than FR at equilibrium it results in a rapid depletion of the P pool, whereas FR maintains a lower φ value, but retains a higher P concentration.

A gradual shift of the FR-HIR to a RL-HIR upon de-etiolation is explained by similar reasoning. After prolonged irradiation the pool of labile P (*l*P) is depleted due to destruction of Pfr. The remaining P is generally considered as more stable. The existence of a small pool of stable P (*s*P) has been indicated by destruction kinetics (Jabben *et al.*, 1980; Heim *et al.*, 1981; Brockman and Schäfer, 1982) as well as by immunological studies (Vierstra and Quail, 1986). More details are given in Chapter 4. Where Pfr destruction is absent or in balance with P synthesis the effect

will be a shift of the HIR maximum towards wavelengths establishing a high φ value, i.e. towards RL.

This explanation of the FR-HIR peak of effectiveness is now generally accepted, but clarification of the fluence-rate dependency often observed, remains so far unresolved. If the HIR depended exclusively on the maintenance of a specific φ value, it should be independent of fluence rate. The equation for photoequilibrium maintained *in vitro* after saturating irradiation (Butler *et al.*,1964; Butler,1972),

$$\varphi=[Pfr]/[P] = \epsilon_r \Phi_r/(\epsilon_r\Phi_r+\epsilon_{fr}\Phi_{fr})$$

(where ϵ_r and ϵ_{fr} are the extinction coefficients of Pr and Pfr, Φ_r and Φ_{fr} are the quantum efficiencies for photoconversion of Pr and Pfr) and data of others (Smith, 1975; Hayward, 1984; Kelly and Lagarias, 1985; Frankland, 1986) indicate that φ is a function of the wavelength and independent of fluence rate. However, an extensive amount of data clearly indicate a fluence-rate dependency of the HIR (e.g. cucumber: Meyer, 1958a; Meyer, 1959; Meyer and Engelsma, 1965; Gaba and Black, 1985b; Thomas and Dickinson, 1979; tomato: Meyer, 1959; Thomas and Dickinson, 1979). One suggestion is that there are one or more dark (thermal) reactions which modify Pfr (Jabben *et al.*, 1982). If these dark reactions are sufficiently rapid, then the photoequilibrium is never reached, because Pfr is removed by dark reactions nearly as fast as it is formed by photoconversion from Pr. Thus the amount of P in the Pfr form will depend on the rate of photoconversion of P, i.e. on the fluence rate.

Several authors state, that Pfr alone can not account for all aspects of the HIR. It is necessary to hypothesize an additional feature, which is a function of the rate of photoconversion of Pr to Pfr. It might act either with Pfr (Schäfer, 1975; Mohr *et al.*, 1979; Johnson and Tasker, 1979; Wall and Johnson, 1983) or independently of Pfr (Smith, 1970). A general characteristic of most models designed to explain the fluence-rate dependency of the HIR is the importance of the rate of photochemical turnover (**cycling**) between Pr and Pfr which is a function of the fluence rate.

What is also difficult to explain on the basis of P alone is the effectiveness of BL in HIR's. Irradiation with BL establishes a specific φ value ($\approx$ 0.4), because both Pfr and Pr absorb to some extent in the BL

spectral region, as can be seen in the absorption spectra of Pfr and Pr. However, the inhibition of hypocotyl elongation of de-etiolated cucumber seedlings by continuous BL is not the same as measured with light of other wavelengths, maintaining a similar φ value (Gaba *et al.*, 1984). If only P was involved, equal φ values should produce equal responses irrespective of the wavelengths used. However, adding BL to a background of high irradiance yellow light (Thomas and Dickinson, 1979) or RL (Attridge *et al.*, 1984), which is predicted to have no significant effect on the φ value maintained, has a marked inhibitory effect on growth. In both cucumber and tomato seedlings the inhibition of hypocotyl elongation by supplementary BL was related to its fluence rate. The inevitable conclusion is that there is co-action in the BL region between P and a separate BL/UV-absorbing photoreceptor (cryptochrome).

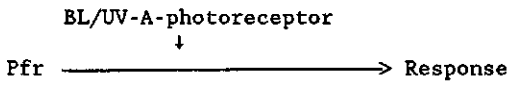
Growth inhibition due to the BL/UV-photoreceptor may be distinguished from those mediated by P by several criteria. (i) BL acts directly upon the hypocotyl of both etiolated and de-etiolated cucumber seedlings, whereas in de-etiolated seedlings the influence of RL is predominantly mediated through light absorption by the cotyledons, which relay the signal to the hypocotyl below (Black and Shuttleworth, 1974; Cosgrove, 1981). (ii) Whereas very low fluence rates of RL are capable of inhibiting hypocotyl elongation of de-etiolated cucumber seedlings, fluence rates of BL lower than 3 μmol m^{-2} s^{-1} fail to inhibit (Attridge *et al.*, 1984). Adding FR to low fluence rate BL (just above this threshold) decreases the inhibitory effect of BL, suggesting that some Pfr is necessary for inhibition by BL (Gaba *et al.*, 1984). This can be explained by the so called 'Presence Theory' (Gaba and Black, 1987). This theory states that the BL/UV-A-photoreceptor requires the presence of a particular amount of Pfr (or a particular φ value) for its action to be expressed; high φ values would enhance the effectiveness of BL.

However, the alternative explanation that BL enhances the effectiveness of P cannot be eliminated. This would result in additional growth inhibition when BL is added to a background irradiation establishing a high φ value and also when irradiation with RL is preceded by BL, if the 'effectiveness amplification' can be preserved after the end of the BL irradiation. Observations in favour of this explanation were made by Meyer (1958a). The increase of hypocotyl length of etiolated cucumber seedlings in BL (several days) after pretreatment with RL (48 h; 16 h RL/8 h D cycles) was similar to the increase of hypocotyl length of seedlings irradiated with RL alone. Seedlings pretreated with 48 h BL followed by RL were inhibited

more than seedlings irradiated with BL or RL alone. This indicates that the inhibitory effect of RL is increased by the preceding BL irradiation. This observation can be explained by the so called 'Responsiveness Theory' (Gaba and Black, 1987). This theory states that the only effect of BL is to establish and maintain responsiveness to P.

Presence Theory

Responsiveness Theory



3.2 Germination

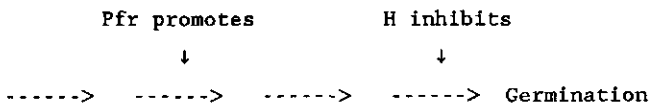
Some seeds have an absolute light requirement for germination, whereas others germinate in complete D. Both tomato and cucumber are examples of species whose seeds exhibit a high dark germination over a broad temperature range (Mancinelli *et al.*, 1966; Yaniv *et al.*, 1967). This is possibly due to the relatively high level of residual Pfr in the dry seeds: in tomato ≈ 40% of the spectrophotometric detectable P is Pfr (Mancinelli *et al.*, 1967) and in cucumber ≈ 75% (Spruit and Mancinelli, 1969). This conclusion is supported by the fact that when P in cucumber seeds is converted predominantly to the Pr form by irradiation of the ripe fruits with FR, the dark germination of seeds from these fruits, that have been subsequently dried in D, is strongly reduced (Gutterman and Porath, 1975). Both tomato and cucumber seed germination can be inhibited by light, intermittent or prolonged exposure usually being necessary. However, temperature plays an important role in determining the effectiveness of a light treatment.

In many dark-germinating seeds studied Pfr has a promotive effect on germination (Cone and Kendrick, 1986). Irradiation with light pulses establishing low φ levels usually inhibit germination, whereas high φ levels fail to inhibit.

At suboptimal temperatures germination of tomato seeds can be inhibited

by a single FR pulse (establishing a low φ level) and the effect is reversed by a RL, BL or WL pulse (all producing a high φ level) applied immediately after FR (Mancinelli *et al.*, 1966; Mancinelli *et al.*, 1967; Yaniv and Mancinelli, 1968). At temperatures about 25 °C or higher the number of FR-inhibited seeds in a population decreases. At these high temperatures germination of tomato seeds can no longer be inhibited by a single FR-pulse (Mancinelli *et al.*, 1967). According to Yaniv *et al.* (1967) germination of cucumber seeds can not be inhibited by a single FR pulse, but Frankland (1986) reported that this inhibition is possible if the FR pulse is given at a suboptimal temperature (15 °C) within 40 h of sowing. Suzuki and Takahashi (1969) even managed to inhibit germination at 25 °C, but only when the FR pulse was applied during the period 16-18 h after sowing. It is possible that Pfr action at 15 and 25 °C is complete after 40 and 18 h D, respectively. Therefore germination is no longer inhibited by removal of Pfr after these time intervals.

During prolonged irradiation experiments germination is still controlled by P, but whereas the low fluence response (LFR), induced by short-term light treatments, appears to be controlled by Pfr levels (φ) alone, under prolonged irradiation the fluence-rate dependent P cycling rate (H), plays an additional important role. This dual action of light may be represented as photocontrol at two steps in the sequence of events leading to germination, one promoted by Pfr, the other blocked by an inhibitory photoreaction, dependent on the rate of P interconversion or cycling (Bartley and Frankland, 1982; Frankland, 1986). In addition, the latter reaction appears to be able to re-induce a Pfr requirement in cases where the Pfr promotive reaction appears to be complete, i.e. where the seeds have escaped FR reversibility.



Prolonged irradiation with FR inhibits germination of tomato and cucumber seeds both at high and sub-optimal temperatures. However, at temperatures above 20 °C only very narrow band FR (730 nm), establishing a very low φ value (<0.02), can inhibit germination of cucumber seeds (Eisenstadt and

Mancinelli, 1974). The Pfr level required to induce a particular germination percentage appears to be lower at higher temperatures.

Whereas short irradiation with RL, BL or WL promotes germination after a FR pulse, exposure to prolonged irradiation causes an inhibition or at least a delay of germination of tomato (Yaniv and Mancinelli, 1968) and cucumber (Eisenstadt and Mancinelli, 1974). This inhibition of germination can be (partially) alleviated in subsequent D, but after prolonged FR, a RL pulse is required for promotion of germination. Apparently, during prolonged irradiation with RL, BL or WL conditions are unfavourable for germination (the high H outweighing the stimulative effect of the relatively high φ level maintained), but in subsequent D germination occurs because of the relatively high residual φ value. After prolonged FR irradiation φ is low and germination can only be attained by a pulse of light establishing a high φ value.

The possibility remains that photo-inhibition of germination by BL involves a pigment other than P. Frankland (1986) reports that in cucumber, BL has an inhibitory effect greater than would be predicted from the φ value it maintains. However, this 'extra' effect disappears when BL is mixed with RL to give a high φ value.

3.3 Anthocyanin synthesis

The biosynthesis of anthocyanins in plant tissues either requires light or is enhanced by it (Beggs *et al.*, 1986). In response to short irradiations (minutes) with RL small amounts of anthocyanin are formed in several species, e.g. cabbage, rye (Mancinelli, 1984a; 1984b; Mancinelli and Rabino, 1985) and mustard (Lange *et al.*, 1971). The effect of a single RL pulse can be reversed by a FR pulse applied immediately afterwards, indicating that P is involved. The extent of the RL/FR reversible response is small, nevertheless significant (Mancinelli, 1985), when compared to the extent of the response brought about by prolonged irradiations. However, no inductive, RL/FR reversible anthocyanin production has been observed in dark-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 P, can be observed in these seedlings. The light-dependent enhancement of the inductive RL/FR

reversible response is affected by: (i) the duration and spectral quality of the light pretreatment, (ii) the duration of the dark interval between the end of the pretreatment and the application of the light pulse, and (iii) seedling age (Mancinelli, 1985).

Whereas short irradiations are only capable of inducing the synthesis of a small amount of anthocyanin, the synthesis of large quantities requires prolonged exposures to high fluence rates. The general characteristics of this response to prolonged irradiation are those typical of the HIR: (i) requirement of prolonged exposures (hours to days) to high fluence rates of visible and near visible light, (ii) the extent of the response is a function of duration and fluence rate of the irradiation, and (iii) the response does not show RL/FR reversibility (Downs, 1964; Hartmann, 1966; Lange *et al.*, 1971; Mancinelli, 1985). The full expression of the HIR requires prolonged exposures, but not necessarily continuous irradiation. Intermittent light treatments were also very effective in inducing anthocyanin synthesis. Irradiations given as cyclic treatments demonstrate RL/FR reversibility, the extent of which is a function of the duration of the dark interval between successive irradiations (Mancinelli *et al.*, 1974; Mancinelli and Rabino, 1975; 1985). This type of treatment indicates the involvement of P.

The spectral sensitivity of anthocyanin synthesis under prolonged irradiation is different in different species. Three main groups of HIR spectral sensitivity can be distinguished (according to Mancinelli (1983)). Group I: UV/BL, RL and FR are all effective in inducing anthocyanin synthesis, even though the relative effectiveness of the different spectral regions may be different in different systems. Examples are mustard and red cabbage (Mohr and Drumm-Herrel, 1981; 1983; Sponga *et al.*, 1986; Mancinelli, 1984b). Group II: UV/BL and RL are active; the effect of FR is negligible. Examples are apple skin sections, leaf disks of red cabbage and *Spirodela polyrrhiza* (Mancinelli, 1977, 1980; Siegelman and Hendricks, 1958). Group III: The UV/BL region is the only active region. Examples are sorghum and wheat (cv. Schirokko) seedlings (Drumm-Herrel and Mohr, 1981; Mohr and Drumm-Herrel, 1983). The spectral sensitivity of anthocyanin synthesis in some other systems appears to be intermediate between these groups. For example, in seedlings of tomato and rye maximum action is in the UV/BL region as in group III, but RL and FR are also effective, although much less than UV and BL (Drumm-Herrel and Mohr, 1982a; 1982b; Mancinelli and Schwartz, 1984).

Apart from enhancing the inductive, RL/FR reversible anthocyanin synthesis, light pretreatments also influence subsequent 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 dark- and light-grown seedlings is the consequence of changes in the relative amount of *IP* to *sP*, as indicated by results obtained in studies of the effectiveness of intermittent light treatments on HIR anthocyanin synthesis (Mancinelli and Rabino, 1985). P action in dark-grown seedlings might be mainly due to *IP* which is lost rather rapidly by destruction and would have only limited time to act. In light-grown seedlings a larger proportion of P action might be exercised by *sP* which is relatively stable and 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 dark intervals between successive irradiations are more pronounced in dark-grown seedlings than in light-grown ones, where action would be expected mainly due to *IP* and *sP*, respectively (Mancinelli and Rabino, 1985).

As discussed earlier in this chapter (3.1.2) it is difficult to explain the effectiveness of BL in HIR's on the basis of P alone. Results of experiments based on the 'light-equivalence principle' have indicated that cryptochrome is involved in the photoregulation of anthocyanin synthesis by BL in tomato seedlings (Sponga *et al.*, 1986, Drumm-Herrel and Mohr, 1982a). There was a significantly higher anthocyanin synthesis in tomato seedlings after treatments with BL than after treatment with light from a RL+FR source producing a similar P photoequilibrium and cycling rate ($\varphi_{BL} = \varphi_{RL+FR}$; $k_{BL} = k_{RL+FR}$ [where k is the P cycling rate]). This indicates that cryptochrome as well as P play a role in this response.

The effect of the pretreatments has been interpreted as an increase in responsiveness to Pfr (Drumm-Herrel and Mohr, 1982a; Oelmüller and Mohr, 1984). This responsiveness amplification can be mediated by P, a BL/UV-photoreceptor (cryptochrome) or a UV-B photoreceptor. Oelmüller and Mohr (1985) proposed a model to describe the mode of co-action between BL/UV and light absorbed by P in the light-mediated synthesis of anthocyanin in sorghum seedlings (Fig. 3.1). Pfr is suggested to be the terminal effector involved in the photoregulation of anthocyanin synthesis, whereas the cryptochrome-mediated process is required to establish responsiveness towards Pfr. The interaction between cryptochrome and P in the photoregulation of anthocyanin varies between species. In tomato it appears

that this interaction is not obligatory, since pretreatment with RL or FR, absorbed by P and not by cryptochrome, is also effective. When continuous irradiation was applied to induce anthocyanin formation in tomato seedlings without an inductive pulse at the end of the light treatment, BL, UV-A and UV-B exerted a strong response, whereas FR and RL were only slightly effective (Drumm-Herrel and Mohr, 1982a). It is therefore possible that in tomato BL/UV only functions to enhance the responsiveness to Pfr.

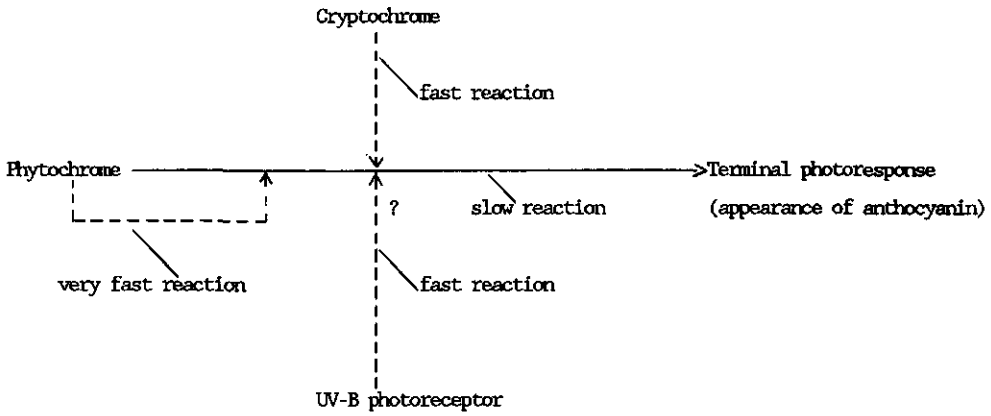


Fig. 3.1 Suggested mode of co-action between blue light (BL)/UV and light absorbed by phytochrome in light-mediated anthocyanin formation in the *Sorghum* seedling. ——> : Temporal sequence of events set in motion by the effector Pfr and leading to the terminal response; --->: Light-dependent reactions which determine the effectiveness of the effector Pfr (i.e. the responsiveness of the anthocyanin-producing mechanism towards Pfr). The point of action of UV-B, relative to the action of BL/UV-A (*via* cryptochrome), remains undecided at present. After Oelmüller and Mohr (1985).

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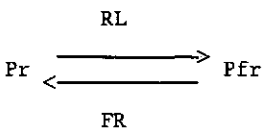
4. PHYTOCHROME

4.1 Introduction

The photochromic photoreceptor pigment phytochrome (P) was postulated by Borthwick *et al.* (1952) on the basis of action spectra (i.e. the relative effectiveness of different wavelengths of light for the induction and reversion of several red (RL)/far-red light (FR) reversible responses). They proposed that phytochrome is synthesized in darkness (D) in the RL-absorbing, inactive form (Pr) which can be phototransformed into the FR-absorbing, active form (Pfr). Upon irradiation with FR, Pfr is photoconverted back to the inactive Pr form. The name phytochrome (meaning 'plant colour') was introduced by W.L. Butler some time later (Borthwick, 1972).



Attempts were made to detect phytochrome by physical means. Seedlings grown in D were first examined, since these lack chlorophyll which would have masked the postulated (blue-green) photoreceptor P. Since P displays photoreversible shifts in absorption, it was possible to detect P in plant tissue by spectrophotometry. Using the absorption maximum of Pr and Pfr (660 and 730 nm respectively) as measuring beams the absorbance differences between the two wavelengths were measured after actinic irradiation with RL and FR. The total P is proportional to the difference in absorbance difference ($\Delta\Delta$ A) between 660 and 730 nm. Alternatively the absorbance differences between 730 and 800 nm can be monitored to prevent interference from other pigments (e.g. chlorophyll) which absorb in the RL spectral region. However, in this case the absorbance change will only be about half of that obtained with 660 and 730 nm.

From the shape of the action spectra Borthwick *et al.* (1952) suggested that P possessed an open-chain tetrapyrrole chromophore similar to that of the algal photosynthetic antenna pigment C-phycocyanin. This prediction has

been shown to be accurate (Rüdiger, 1986). Fig. 4.1 illustrates the structure of the chromophore, its similarity to C-phycocyanin, the attachment of the tetrapyrrole to the apo-protein and the possible conformational changes that occur upon phototransformation.

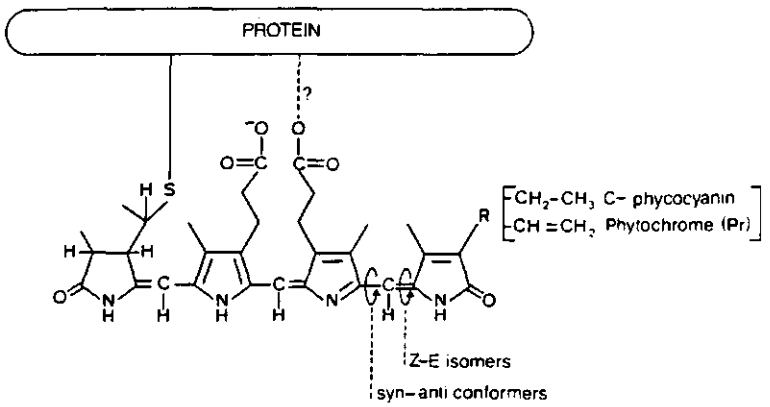


Fig.4.1 The chromophore of phytochrome and C-phycocyanin with the possible types of configurational change that could occur upon phototransformation of phytochrome. (After Kendrick and de Kok, 1983)

Extraction of P in its native state has proved difficult because of the high susceptibility of P to proteolysis by endogenous proteases. The first purifications of P yielded a protein with a molecular mass of 60 kDa ('small' P). Using more rapid techniques and sources low in endogenous proteases P samples with bands corresponding to molecular masses of 114, 118 and 120 kDa were observed. This type of preparation is commonly called '120-kDa' or 'large' P (Gardner *et al.*, 1971; Pratt, 1982). More recently it was discovered that the full-length molecule ('native' P) of *Avena* has a molecular mass of 124 kDa (Vierstra and Quail, 1982, Vierstra *et al*, 1984). However, there appears to be some variability in apparent molecular mass of full-length P from different plant species (Vierstra and Quail, 1986). Biochemical studies indicate that, in the native chromoprotein, the polypeptide is folded into two principal domains: a globular, chromophore-bearing, NH_2-terminal domain and a more open, COOH-terminal domain. These two domains are linked by a proteolytically vulnerable segment (Jones and Quail, 1986; Lagarias and Mercurio, 1985; Vierstra *et al.*, 1984).

Phototransformation-induced conformational changes of the molecule have been indicated by studying the differential sensitivity to proteolysis of the Pr and the Pfr form. A small segment of the NH_2-terminal domain is especially susceptible to cleavage when P is in the Pr form, while a small region in the COOH-terminal domain is more susceptible in the Pfr form (for review see Vierstra and Quail, 1986).

It has been found that at least four genes code for P in etiolated *Avena* seedlings (Hershey *et al.*, 1985). Sequence analysis of the coding region of a cDNA clone from *Cucurbita* enabled a comparison to be made between the phytochromes of these two species (Sharrock *et al.*, 1986). Within a short period the sequence analysis of P from a number of other species should be available, thus enabling identification of highly conserved regions of the molecule (M. Furuya and P.H. Quail, pers. comm.). These data will provide information on conserved structural features of the P molecules of monocotyledons and dicotyledons. Sequences involved in at least two properties of the P molecule are expected to be conserved: (i) those involved in interactions with the chromophore, since the spectral properties of the photoreceptor from diverse sources are highly conserved; and (ii) those involved in the biological 'active site' of the molecule, since it is reasonable to expect that the molecular mechanism of P action will be conserved between species (Quail *et al.*, 1987). The level of sequence homology in the NH_2-terminal two-thirds of the polypeptide is higher than in the COOH-terminal one-third. This suggests that the NH_2-terminal domain is more likely to be involved in critical structural and functional properties of the photoreceptor.

Although spectrophotometric assays are very useful to detect and quantify P they have some serious limitations, e.g. spectrophotometric assays will not detect non-chromophore containing and non-photoreversible P, and the presence of chlorophyll, especially abundant in light-grown plants, drastically alters $\Delta\Delta$ A signals by screening and fluorescence artifacts (Pratt, 1983).

Since P is a protein it is an excellent antigen. Immunochemical assays of P offer several advantages over spectrophotometric assays, including their high sensitivity, their ability to detect spectrally aberrant molecules and their insensitivity to other pigments such as chlorophyll. Their disadvantage is that they generally cannot, at the present time, discriminate between the Pfr and Pr forms.

The current understanding of the chemical properties of P comes mainly

from the study of P extracted from etiolated tissue. There are two principal reasons for this situation. Firstly, chlorophyll in light-grown tissues prevents spectrophotometric assay of P. Secondly, light-grown plants contain one or two orders of magnitude less P than etiolated plants (Pratt and Cordonnier, 1987). However, with the development of methods for purifying P to a high degree and the production of very specific monoclonal antibodies, immunochemical assays increase the possibilities of studying P, especially in light-grown plants.

There have been indications of the existence of more than one type of P from several fields of research. At first these indications originated from hypotheses, attempting to explain apparent paradoxes (so called 'P paradoxes') between physiological and in-vivo spectrophotometric data (Hillman, 1967; Jabben and Holmes, 1983). They suggested that plants contain at least two pools of P: a bulk pool that is inactive, at least with respect to some photomorphogenetic responses, and a quantitatively minor pool that is active. On the basis of physiological (Mancinelli, 1984; Mancinelli and Rabino, 1985) and spectrophotometric evidence (Jabben *et al.*, 1980; Heim *et al.*, 1981; Brockman and Schäfer, 1982; Jabben and Holmes, 1983) it has been proposed, that the 'bulk' of the P in etiolated or re-etiolated seedlings is unstable, whereas the P in light-grown seedlings is relatively stable. Immunological (Shimazaki and Pratt, 1985; Abe *et al.*, 1985; Tokuhisha *et al.*, 1985; Vierstra and Quail, 1986; Konomi *et al.*, 1987) studies have also provided evidence that phytochromes from green and etiolated seedlings are different from each other.

Applying a procedure for rapid elimination of chlorophyll from P-containing extracts to permit spectral assay, it was demonstrated that P from green tissue had a Pr absorption maximum at 4 to 15 nm shorter wavelength than Pr from etiolated tissue (Tokuhisha *et al.*, 1985). Furthermore, the green-tissue molecule is not immunoprecipitated by polyclonal or monoclonal antibodies against etiolated-tissue P and has a proteolytic peptide map distinct from that of etiolated-tissue P (Tokuhisha *et al.*, 1985; Shimazaki *et al.*, 1985). However, it is not yet known whether the green-tissue molecule is a gene product different from that in etiolated tissue or the same gene product that has been subsequently modified (processed).

There is as yet no agreed terminology for the different types of P. The bulk pool of P has been described as labile (*lP*) (Jabben and Holmes, 1983; Schäfer *et al.*, 1984) or type I P (Smith and Whitelam, 1987; Nagatani *et*

al., 1987; Konomi *et al.*, 1987), whereas the P from light-grown plants, proposed to be stable, has been described as light stable (*s*P) or type II P. In addition etiolated(-oat) P and green(-oat) P has been used by several workers (e.g. Pratt and Cordonnier, 1987; Tokuhisha *et al.*, 1985). However, the terms etiolated- and green-P are confusing, since both etiolated and light-grown tissue contain both P types. For example, in embryonic axis of pea Konomi *et al.* (1987) detected P I and II after 12 h incubation in D (ca. 0.2 and 0.05 μg/axis respectively) and also P I and II after 12 h incubation in the light (both ca. 0.05 μg/axis).

Although evidence has been presented that two types of P exist in both etiolated and light-grown seedlings, it is very difficult to discriminate between responses controlled by *I*P or *s*P, since both are present and their absorption spectra are practically indistinguishable (Pratt and Cordonnier, 1987). Action spectra for responses controlled by both would therefore be similar. Photoreceptor mutants, lacking one of the P types or its function, could provide a useful tool to ascribe a response to one of the P types. In this chapter the P contents of dark- and light-grown plants of two possible P photoreceptor mutants and their isogenic wild types are presented.

4.2 Materials and methods

4.2.1 Plant material

The long hypocotyl mutants of cucumber (*lh*) and tomato (*au*) and their isogenic wild types, as well as the 'high-pigment' mutant of tomato (*hp*) used in the present experiments have been described in Chapter 2.

4.2.2 Dual-wavelength assay of phytochrome

For measurements of the P content of etiolated and de-etiolated tissue a custom-built dual-wavelength spectrophotometer was used with the measuring beam set at 730 and the reference beam at 806 nm (Spruit, 1970). The photoreversible difference in absorbance difference ($\Delta\Delta$ A) following saturating alternate actinic RL and FR irradiation is proportional to the total P present. The actinic light consisted of a 250-W quartz-iodide projection lamp filtered through interference filters (Balzer B40 type, Balzer Liechtenstein), 10 nm half band width at 50% of the transmission maximum.

4.2.3 Difference spectra of phytochrome photoconversion

The difference spectra for P phototransformation were determined using an Aminco DW-2a spectrophotometer equipped with Midan analyzer. Initially the cuvettes were irradiated with saturating RL (660 nm). The spectrum was scanned and recorded into the Midan analyzer. One cuvette was then irradiated with saturating FR (730 nm) and the spectrum again scanned and recorded. The two spectral scans were then subtracted, amplified and plotted to reveal the difference spectrum for P phototransformation. The actinic light has been described in 4.2.2.

4.2.4 Phytochrome measurement in light-grown tissues.

Although P spectrophotometry is not feasible with green leaves, flower petals often have sufficiently low levels of chlorophyll to enable measurement (Spruit, 1971). P measurement is also possible in light-grown tissue bleached with the herbicide Norflurazon. Bleaching is a consequence of the inhibition of carotenoid biosynthesis and the resultant photobleaching of chlorophylls (Rombach *et al.*, 1982). This technique is therefore usually restricted to young seedlings that have sufficient food reserves to sustain growth. The strategy adapted in this study was to grow plants until they had several mature photosynthetic leaves and then selectively treat the apex with Norflurazon.

4.2.5 Sample preparation

For determination of difference spectra seeds were spread out on moistened filter paper in Petri dishes in D at 25 °C. After a specific D interval either seeds or seedlings were packed into 2 cuvettes (path length 5 mm) and cooled to 0 °C.

The P content of etiolated tissue was measured in intact dark-grown hypocotyls and roots (tomato) or 5 mm segments of hypocotyls (cucumber) packed into cuvettes with path length 6 mm for tomato and 2.3 mm for cucumber. For the assay of P in flower petals, the petals were removed from plants (both cucumber and tomato) growing in a greenhouse during the summer and immediately packed into the cuvettes (path length 2 mm). For measurement in bleached leaves, plants (both cucumber and tomato) were initially grown in the phytotron under a WL/D regime of 16 h WL (8 W m^{-2} [PAR, 36 μmol m^{-2} s^{-1}])/ 8 h D at 20 °C. After the plants were about 6 weeks old the tips of the plants were dipped into a solution of 10^{-5} *M* Norflurazon (Sandoz AG, Basel) twice a week, until the new leaves were devoid of chlorophyll. A

uniform sample of leaves was selected for wild type and mutant, cut into small pieces and packed into the cuvettes (path length 2 mm).

4.3 Results and discussion

4.3.1 Phytochrome in dark-grown cucumber *lh* mutant and wild type.

The wild type and the *lh* mutant have equal amounts of spectrophotometrically detectable P in seeds (Fig. 4.2). The increase in P

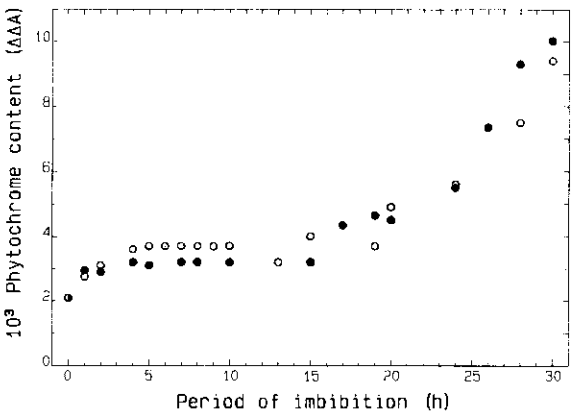


Fig.4.2 The phytochrome (P) content of seeds during imbibition at 25 °C expressed as ΔΔ $A_{730\text{-}800\ nm}$ of wild type (o) and *lh* mutant (●). Demonstrates similar P content and apparent P synthesis in the *lh* mutant and its wild type.

level after 15 h D incubation and preceding germination indicates that apparent synthesis of P in wild type and the *lh* mutant is similar. In a few experiments hypocotyls of dark-grown seedlings of the wild type and the *lh* mutant were measured. The results showed similar high levels of P (Table 4.1). Difference spectra for P phototransformation are also identical (Fig. 4.3). Apparently there is no difference in bulk *lP* between wild type and *lh* mutant.

Table 4.1. Phytochrome content of etiolated and de-etiolated tissue of cucumber. Note that, since the samples have different scattering properties, comparison should only be made between wild type and mutant for each particular tissue.

Phytochrome content (10^3 $\Delta\Delta$ $A_{730-800\ nm}$ ± S.E.)		
	Wild type	*lh* mutant
Etiolated seedlings	3.92	4.10 ± 0.10
Bleached leaves	1.30 ± 0.07	0.42 ± 0.06
Flower petals	0.78 ± 0.06	0.37 ± 0.04

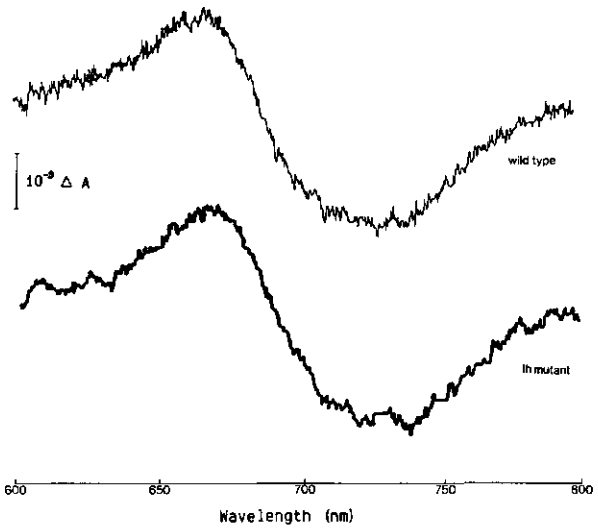


Fig.4.3 Difference spectrum for phytochrome photoconversion (far-red irradiated minus red irradiated) in dark-grown seedlings of the cucumber *lh* mutant and its wild type. No difference is observed between wild type and mutant.

4.3.2 Phytochrome in light-grown cucumber *lh* mutant and wild type.

Figure 4.4 and Table 4.1 demonstrate that the P content of both flower petals and bleached leaves in the *lh* mutant is about 35-50% of that in the wild type. The difference between *lh* mutant and wild type could be

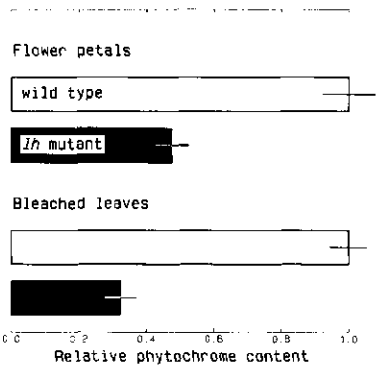


Fig.4.4 Phytochrome measurements in flower petals and Norflurazon-bleached leaves of light-grown cucumber plants. Demonstrates that the level of phytochrome is reduced in the *lh* mutant compared to the wild type in light-grown tissues. The actual $\Delta\Delta$ $A_{730-800\ nm}$ in wild type flower petals and bleached leaves were 0.78 ± 0.06 x 10^{-3} and 1.30 ± 0.07 x 10^{-3} respectively.

attributed to *s*P being absent in the *lh* mutant. The detectable P in the *lh* mutant is proposed to be the steady state level of *l*P. Only a minor pool of *s*P is thought to be present in etiolated seedling. The proposed absence of *s*P would therefore be impossible to detect in the presence of the large background pool of *l*P. The reported spectral differences between *l*P and *s*P lie in the red region of the spectrum (Tokuhisha *et al.*, 1985). Since P level is so low and the traces of chlorophyll and their reactions preclude measurement in the red spectral region, measurements were restricted to 730 *v* 800 nm. The conclusion about the absence of *s*P in the *lh* mutant is therefore only tentative and confirmation will await the availability of specific antibodies against *s*P and *l*P for cucumber.

4.3.3 Phytochrome in tomato *au* mutant and wild type.

Previous measurements of Koornneef *et al.* (1985) and those presented in Table 4.2 using dual-wavelength spectroscopy indicated that the *au* mutant is deficient in photoreversible absorbance changes attributable to P or possesses a pigment with an altered spectrum. Dual-wavelength analysis cannot discriminate between these two possibilities. The *in vivo* difference spectra presented in Figure 4.5 demonstrate that etiolated seedlings of the *au* mutant contain less than 5% (which is the detection limit) of the P level

found in the wild type. This is in agreement with immunochemical data of Parks *et al.* (1987), which indicate that etiolated seedlings of the *au* mutant are deficient in a major immunodetectable protein (116 kDa) normally present in the wild type. It has been demonstrated that this protein has the properties of phytochrome.

Table 4.2. Phytochrome content of etiolated and de-etiolated tissue of tomato. Note that since the samples have different scattering properties comparison should only be made between wild type and mutant for each particular tissue.

Phytochrome content (10^3 $\Delta\Delta$ $A_{730\text{-}800\ nm}$ ± S.E.)			
	Wild type	*au* mutant	*hp* mutant
Etiolated seedlings	7.98 ± 0.17	0.00	6.56 ± 0.50
Bleached leaves	0.95 ± 0.10	0.56 ± 0.03	0.87 ± 0.12
Flower petals	0.50 ± 0.04	0.28 ± 0.03	nd

nd = not determined

A mutation affecting the production of mRNA coding for P could result in the observed low P level. However, Sharrock *et al.* (1988) found no evidence for abnormal transcription of the P gene and they found that fully functional P mRNA is present in the *au* mutant. Another explanation of the P deficiency could be an abnormal instability of the synthesized protein. This instability could be the result of (i) an alteration of a critical site or region in the polypeptide chain so that it is degraded by the protein turnover system of the cell, (ii) an abberation in the chromophore biosynthetic pathway resulting in the synthesis and attachment of an altered chromophore could induce instability of the molecule, (iii) due to an aberration in the chromophore, protein, or chromophore attachment step the chromophore has not been attached and the incomplete molecule is unstable. Evidence for pea and oat in which gabaculine, an inhibitor of chromophore synthesis, was employed, argues against (iii) and indicates that the chromophoreless polypeptide is stable (Jones *et al.*, 1986; Konomi and Furuya, 1986; Elich and Lagarias, 1986). Finally, a specific degradation pathway for P could have been altered so that the pigment is always degraded

within the cell. However, this explanation seems unlikely, since an altered degradation pathway would undoubtedly lead to degradation of other proteins and cause several other lesions in the mutant. In tomato there also appear

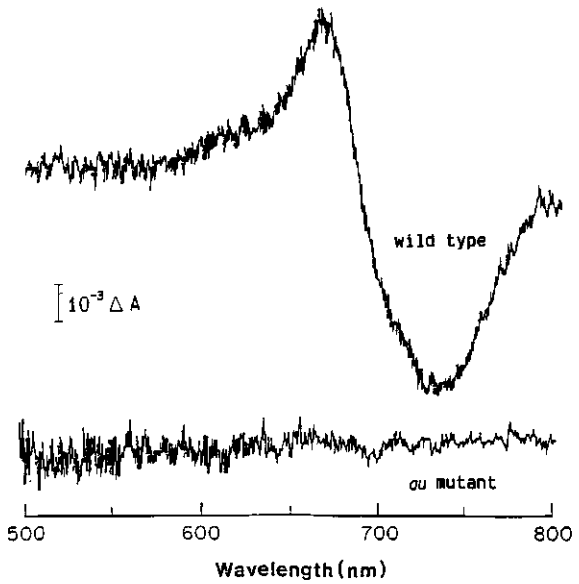


Fig.4.5 Difference spectrum for phytochrome photoconversion (far-red irradiated minus red irradiated) in dark-grown seedlings of the tomato *au* mutant and its wild type. No phytochrome is detectable in the mutant.

to be multiple genes coding for P. One has been mapped to chromosome 10, whereas the *au* locus is situated on chromosome 1 (Koornneef *et al.*, 1986; Sharrock *et al.*, 1988). While the other genes have yet to be mapped it seems unlikely that the *au* lesion is the result of a single amino acid mutation of the protein.

The spectrophotometrically determined P content of both flower petals and bleached leaves in the *au* mutant is about 40-50% of that in the wild type (Table 4.2 and Fig. 4.6). The difference between *au* mutant and wild type could be attributed to *l*P being absent in the *au* mutant. The detectable P in the *au* mutant is proposed to be the level of *s*P. However, as with the *lh* mutant of cucumber described above, the conclusion is only tentative and confirmation will await the availability of specific antibodies against *s*P and *l*P. Attempts using antibodies raised against other species have so far failed to detect P in light-grown tomato (B.M. Parks and P.H. Quail, pers. comm.).

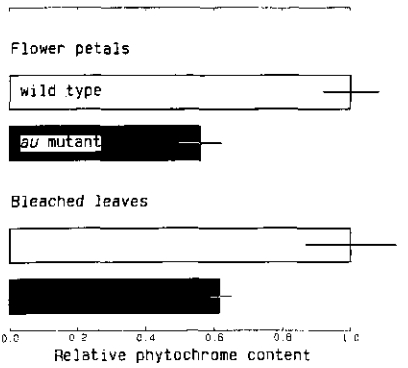


Fig.4.6 Phytochrome measurements in flower petals and Norflurazon-bleached leaves of light-grown tomato plants. Demonstrates that the level of phytochrome is reduced in the *au* mutant compared to the wild type in light-grown tissues. The actual $\Delta\Delta A_{730\text{-}800\ nm}$ in wild type flower petals and bleached leaves were $0.50 \pm 0.04 \times 10^{-3}$ and $0.91 \pm 0.12 \times 10^{-3}$ respectively.

4.3.4 Phytochrome in the tomato *hp* mutant.

Etiolated seedlings as well as light-grown, and Norflurazon bleached, leaves of tomato wild type and the *hp* mutant appear to have equal amounts of spectrophotometrically detectable P (Table 4.2). The consequences of these results will be discussed in Chapter 8 in relationship to anthocyanin synthesis.

Acknowledgements

We thank Sanodoz AB Basel for the gift of a sample of Norflurazon.

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5. LONG TERM GROWTH EXPERIMENTS WITH CUCUMBER

5.1 Introduction

Cucumber seedlings have been used extensively in the study of plant photomorphogenesis (Chapter 3). Both phytochrome (P) and a specific blue light (BL)-absorbing photoreceptor (cryptochrome) are involved in the regulation of development (Thomas and Dickinson, 1979; Cosgrove, 1981; Drumm-Herrel and Mohr, 1982; Gaba and Black, 1985b). There appears to be interaction between these photoreceptor systems, but the nature of this interaction has not yet been fully elucidated. Experiments to date have been equivocal, making it impossible to quantify the relative importance of the two photoreceptors. Mutants which lack one photoreceptor or its action would provide a useful means of estimating the nature of the photoreceptor interaction. Photomorphogenetic mutants have been described for both *Arabidopsis* (Koornneef *et al.*, 1980) and tomato (Koornneef *et al.*, 1985; Chapter 2). These mutants are characterized by their long hypocotyls and pale green leaves when grown in white light (WL). Some have reduced germination, reduced photo-induction of anthocyanin synthesis, and no spectrophotometrically detectable P in etiolated seedlings. The phenotype of some of the *Arabidopsis* and tomato mutants is consistent with them being deficient in P and/or its action.

Recently, mutants of cucumber were identified which also have long hypocotyls in WL (Robinson and Shail, 1981; Koornneef and van der Knaap, 1983). Although less extreme than the *Arabidopsis* and tomato mutants under glasshouse conditions, this long hypocotyl (*lh*) mutant of cucumber exhibits an apparent reduced chlorophyll content compared to the wild type.

In Chapter 4 it was shown that wild type and *lh* mutant seeds have equal amounts of spectrophotometrically detectable P. The increase in P level after 15 h dark (D) incubation, yet preceding germination, indicates that apparent D synthesis of P in wild type and the *lh* mutant is similar. Spectrophotometric measurements of P in flower petals and Norflurazon-bleached leaves indicated that light-grown tissues contain 35-50% of the P level in the wild type (Chapter 4). The P detectable in light-grown plants of the *lh* mutant was proposed to be labile P (*l*P) and the difference between mutant and wild type P levels represents the level of light-stable P (*s*P) in WL. The difference between responses of *lh* mutant and wild type will

therefore reflect the quantitative role of the P deficient in the *lh* mutant. In this chapter the results of experiments concerning elongation growth of the *lh* mutant and its isogenic wild type are presented and the possible role of the different P types in several elongation growth responses is discussed.

5.2 Materials and methods

5.2.1 Plant material

The long hypocotyl (*lh*) mutant of cucumber used in the present experiments has been described in chapter 2.

5.2.2 Light sources.

A description of the light sources used in these experiments can be found in appendix 'Light sources'. The fluence rates used are given in the figure legends.

5.2.3 Screening under broad spectral band light sources

Seeds of wild type and *lh* mutant were sown in transparent plastic boxes (10x10x8 cm) on 5 layers of filter paper saturated with distilled water, and placed in D for 3 d for germination at 25 °C. After this time 10 seedlings were selected per box with approximately the same hypocotyl length, 2 boxes being used for each treatment. Seedlings were transferred to continuous UV-A, BL, RL, and FR. The D controls were kept in wooden boxes covered with black polythene and were placed in each of the light cabinets. In a second series of experiments seedlings were de-etiolated by a period of 8 h WL before transfer to the broad-band cabinets. The hypocotyls were measured daily under a dim green safelight, using a ruler.

5.2.4 Seedling growth in continuous light

Seeds of wild type and *lh* mutant were sown in 10 cm diameter plastic flower pots filled with potting compost and incubated in D for 3 d at 25 °C. Seedlings of approximately the same hypocotyl length of both wild type and *lh* mutant were selected prior to the onset of irradiation. At least 20 seedlings were used for each treatment. The length of each hypocotyl was measured daily with a ruler over a period of several days. In addition, epidermal cell lengths of plants grown in continuous D, WL or RL were

estimated as described in 5.2.5. At the end of the experiments the following parameters were determined: the area of cotyledons and leaves; the fresh and dry weights of the hypocotyls, cotyledons and leaves; and chlorophyll *a* and *b* content of leaves, cotyledons and hypocotyls by the method of MacKinney (1941).

5.2.5 Estimation of cell length

A small amount of quick-hardening resin (Xanthopren) was spread daily on the hypocotyls and peeled off after hardening. The resin contained an impression of the epidermal cells from which an accurate copy was made with polystyrene toluene which could be examined under the microscope. Mean epidermal cell length was estimated by measuring representative cells of 5 to 10 impressions from independent hypocotyls. Wild-type cell lengths were measured in the middle of the hypocotyl. Cell lengths of the *lh* mutant were measured in the lower and upper part of the hypocotyl and then averaged to estimate the mean lengths of the epidermal cells, since cell length appeared to increase linearly between the upper and lower part of the hypocotyl. The estimated number of cells per hypocotyl was obtained by dividing the hypocotyl length by the mean epidermal cell length and is not the actual total number of cells composing the epidermis of the hypocotyl.

5.2.6 Red/far-red reversibility experiments

Seeds of wild type and *lh* mutant were imbibed for 4 h in distilled water, rinsed 3 times in tap water and sown in moistened vermiculite. After 90 h at 25 °C in D etiolated seedlings were selected for approximately the same hypocotyl length (7-9 cm). De-etiolated seedlings were obtained similarly, but 90 h after sowing they were transferred to WL for 30 h at 25 °C, before selection for approximately the same hypocotyl length (wild type: 6-7 cm; *lh* mutant: 8-9 cm). At the time of selection the seedlings were transferred to fresh vermiculite. The following daily irradiation schedules were given: D; 5 min RL; 15 min FR; 5 min RL followed immediately by 15 min FR. After 4 cycles the hypocotyl lengths and fresh weights of the cotyledons were measured. These results were then expressed as mean percentage change with respect to the D controls.

5.2.7 Simulated phototropism

De-etiolated seedlings were grown in the same way as described in 5.2.6., except that the WL treatment was extended to 54 h in the case of the

lh mutant. This was necessary to achieve the same developmental stage of the cotyledons as in the wild type. After de-etiolation the seedlings were selected for approximately the same hypocotyl length (wild type: 5-6.5 cm and *lh* mutant: 7.5-9 cm) and transferred to fresh vermiculite. One cotyledon of each seedling was covered with aluminium foil. The seedlings were maintained in D or irradiated with WL, RL or BL from above. After 24 h each seedling was removed from the vermiculite and photocopied. From the photocopies the angle of curvature of each hypocotyl was estimated using a protractor.

5.2.8 Treatments with gibberellin

Seeds of cucumber were imbibed in distilled water for 24 h in D at 25 °C and then transferred for 24 h to a gibberellin (GA_{4+7}) solution (a commercial gibberellin (GA) solution [Berelex, 27 μM GA_{4+7}] diluted to concentrations in the range 10^{-3} to 10^{-6}, as described in the figure legends). This treatment is referred to as a 'seed soak'. The seeds were then sown in plastic boxes (10x10x8 cm) on 5 layers of filter paper saturated with distilled water and placed in D for 2 d for germination at 25 °C. Seedlings used for irradiation with broad band light sources were treated with 10^{-4} GA_{4+7} and irradiated as described under 5.2.3. Seedlings used for the GA dose-response curve were treated as described under 5.2.3, but with a daily irradiation schedule of 8 h WL (8 W m^{-2} [PAR, 36 μmol m^{-2} s^{-1}] and 16 h D.

5.2.9 Presentation of results

Results are expressed as mean ± S.E. of representative experiments and have all been repeated at least once with qualitatively similar results.

5.3 Results and discussion

Both the wild type and the *lh* mutant of cucumber showed similar high dark germination. The reduction in germination observed in some experiments was not related to the genotype but to the seed harvests from individual fruits. Allowing fruits to ripen longer on the mother plant before harvest resulted in consistent high dark germination.

When D-grown seedlings of cucumber were transferred to continuous WL, the difference in hypocotyl growth of the wild type and the *lh* mutant

becomes obvious (Fig. 5.1). Preliminary examination of the cell lengths of the epidermal cells (Fig. 5.1) indicated that the difference in length of the hypocotyls appears to be due to increased cell elongation rather than an increase in cell number.

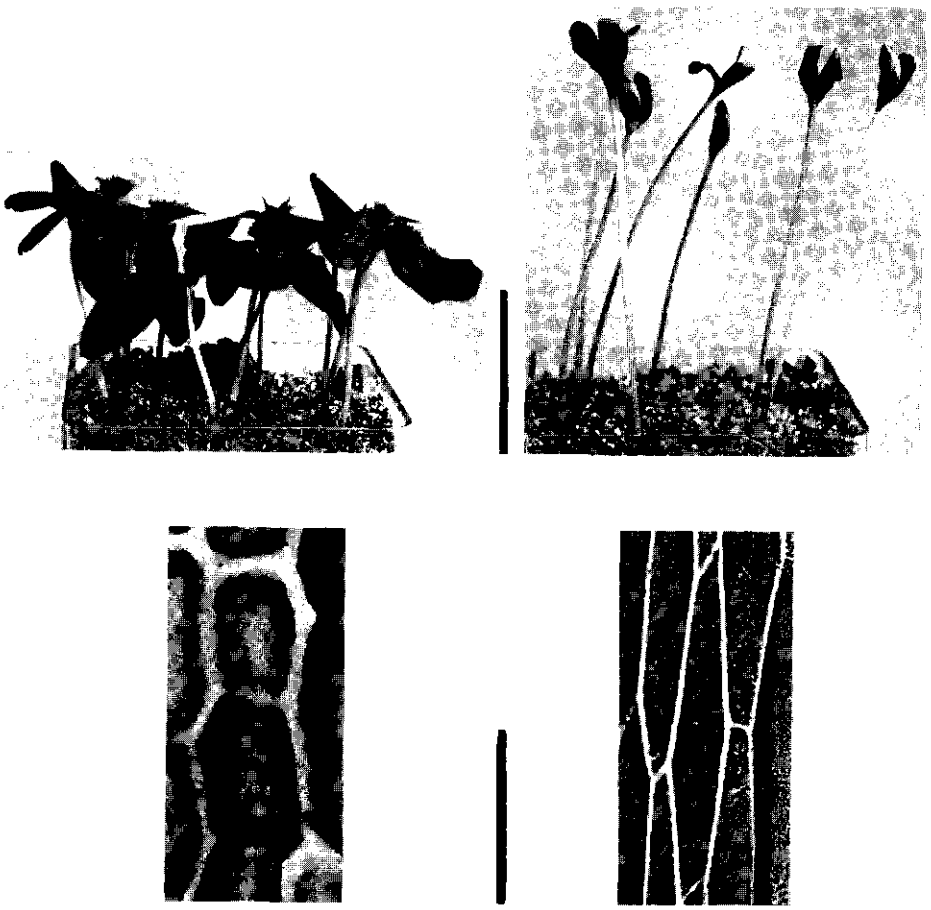

Fig. 5.1 Seedlings of cucumber wild type and *lh* mutant germinated in darkness for 4 d and transferred, when the hypocotyls were in both cases about 4 cm long, to continuous white light (45 μmol m^{-2} s^{-1}) for 7 d at 25 °C. The bar indicates 5 cm. Demonstrates the delayed de-etiolation of the *lh* mutant and its longer hypocotyl compared to its wild type. Below are representative impressions of the hypocotyl epidermal cells, which suggest that the difference in length of the hypocotyls lies predominantly in cell elongation. The bar indicates 0.1 mm.

When germinated in D and transferred, either directly or after de-etiolation by 8 h WL, to continuous UV-A, BL, or FR, hypocotyl growth of the wild type and of the *lh* mutant was very similar (Fig. 5.2). However, in RL, compared

to the wild type, the *lh* mutant shows a significant reduction in hypocotyl inhibition. In continuous D (end-point determination, no safelight used) no difference was observed in hypocotyl growth rates between the wild type and the *lh* mutant. Only the wild type showed a slight inhibition when exposed to green safelight compared to the 'absolute' D control.

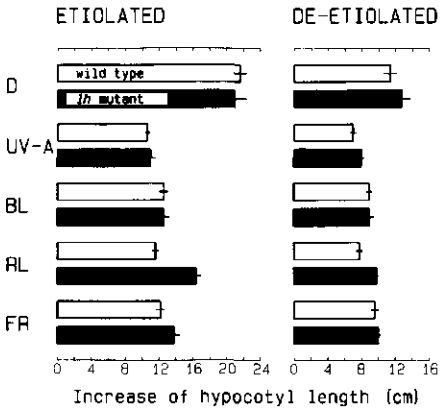

Fig. 5.2 The increase in hypocotyl length ± S.E. in 7 d of etiolated and de-etiolated [pretreated with 8 h white light (WL) of 45 μmol m^{-2} s^{-1}] wild type and *lh* mutant in broad band light sources (3.6 μmol m^{-2} s^{-1} ± 5%); UV-A, blue (BL), red (RL), far-red (FR), or darkness (D). Seedlings were grown for 4 d in darkness before transfer to the light. Demonstrates reduced responsiveness of the *lh* mutant to RL compared to its wild type.

The *lh* mutant showed clear biphasic growth kinetics in WL (Fig. 5.3): an initial rapid growth (about 3 d) followed by an extended linear growth phase. In WL and RL there was only slight inhibition in the case of the *lh* mutant compared to D. The wild type was strongly inhibited in both WL and RL. The epidermal cells of the *lh* mutant were distinctly longer than those of the wild type. The mean cell lengths of epidermal cells of the *lh* mutant

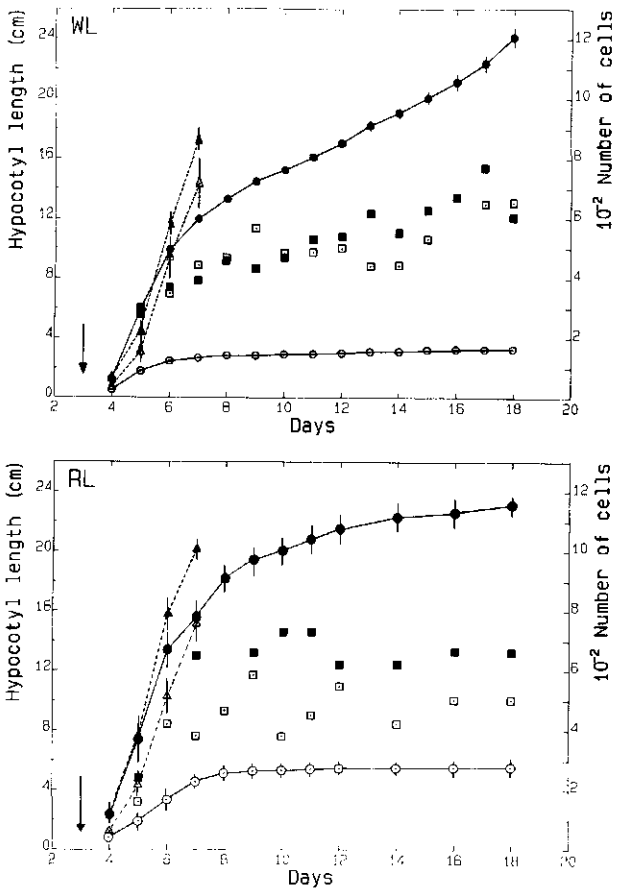

Fig. 5.3 Hypocotyl length ± S.E. in darkness (Δ,▲) and light (o,●), and estimated number of epidermal cell lengths per hypocotyl (□,■) during growth in white light (WL [45 μmol m^{-2} s^{-1}]) or red light (RL [14 μmol m^{-2} s^{-1}]) at 25 °C of cucumber wild type (open symbols) and *lh* mutant (closed symbols). The actual mean cell lengths of the hypocotyl epidermal cells over the time period 8 - 18 d in wild type and *lh* mutant were 6 ± 0.3 and 32 ± 0.1 μm respectively in WL and 11 ± 1 and 32 ± 1 μm respectively in RL. The arrow indicates the onset of continuous irradiation. Demonstrates the initial rapid growth during which cell division occurs and a second growth phase, dominant in the *lh* mutant, which is predominantly a consequence of cell elongation.

and of the wild type were 32 ± 0.1 and 6 ± 0.3 μm respectively in WL and 32 ± 1 and 11 ± 1 μm respectively in RL over the time period 8-18 d. Knowing the mean epidermal cell- and hypocotyl-lengths, an estimate was made of the number of cells per hypocotyl which indicated that the *lh* mutant and the wild type have comparable numbers of cells per hypocotyl (Fig. 5.3). From

these results it is clear that cell division in the *lh* mutant is more or less restricted to the first growth phase whereas the second linear growth phase observed in WL is predominantly cell extension.

The reason for the 10-20 fold difference in hypocotyl length between the wild type and the *lh* mutant that ultimately results in WL does not appear to be a consequence of differences in cell division. The estimation of the number of cells per hypocotyl, although only an approximation, revealed that in all cases during the second phase of growth, irrespective of hypocotyl length, the estimated number of cells per hypocotyl was remarkably constant, falling in the range 500-700 cells. The differences in hypocotyl length between the wild type and the *lh* mutant must therefore be predominantly a consequence of cell elongation. This can be thought of as a prevention or retardation of cell maturation. The Thompson hypothesis (1954; 1959) proposes that RL accelerates all phases of development of plant cell division, enlargement and maturation, resulting in reduced cell numbers and size. It is therefore possible to postulate that it is the last phase of cell maturation which is retarded in the *lh* mutant.

Examination of the chlorophyll content of the cotyledon (on a mg chlorophyll per g fresh weight basis) revealed no difference between the wild type and the *lh* mutant in WL and RL. There was also no difference in chlorophyll *a/b* ratio. In WL a small reduction of chlorophyll content of the leaves of the *lh* mutant compared to the wild type was observed.

Seedlings germinated in D or de-etiolated by 8 h WL were exposed to daily irradiation of a pulse of RL, FR, or RL followed immediately by FR to test for the involvement of P in elongation growth. The results (Fig. 5.4) demonstrate that in the case of the wild type both etiolated and de-etiolated seedlings exhibit P control of hypocotyl elongation and cotyledon expansion. The *lh* mutant, although exhibiting reduced response, clearly has hypocotyl elongation growth and cotyledon expansion controlled by P, although not in de-etiolated seedlings.

The wild type and the *lh* mutant are similar with respect to: (i) rate and percentage of seed germination, (ii) P content of seeds (Chapter 4), (iii) apparent P synthesis in D which precedes germination (Chapter 4), (iv) the hypocotyl growth rate in D and their epidermal cell lengths (Fig. 5.3), (v) hypocotyl inhibition by UV-A and BL (Fig. 5.2), (vi) RL/FR reversibility of hypocotyl growth of etiolated seedlings (Fig. 5.4). The process of de-etiolation appears to be retarded in the *lh* mutant compared to the wild type (compare cotyledon expansion and hypocotyl inhibition in Fig. 5.1).

After de-etiolation the RL response of the *lh* mutant decreases dramatically compared to the wild type.

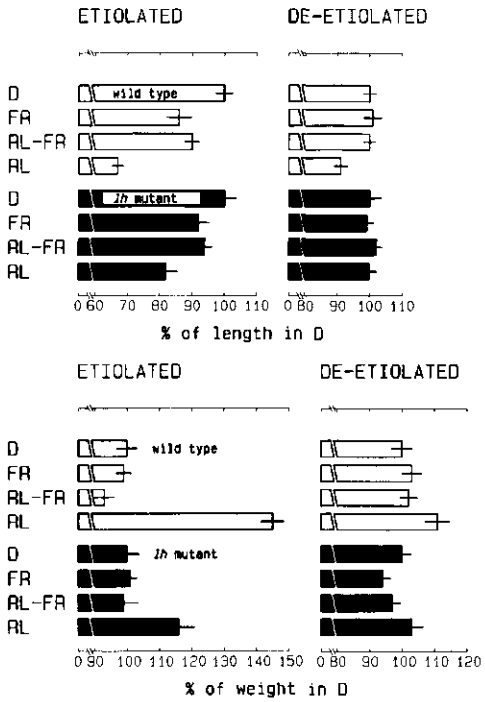

Fig. 5.4 Investigation of red (RL)/far-red light (FR) reversibility of hypocotyl growth inhibition and cotyledon expansion (cotyledon weight) of etiolated and de-etiolated (pretreated with 8 h white light [45 μmol m^{-2} s^{-1}]) wild type and *lh* mutant. The irradiation schedules consisted of 5 min RL and 15 min FR, sufficient to saturate phytochrome phototransformation, and were given every 24 h for 4 d. The seedlings were otherwise maintained in darkness at 25 °C. Results are expressed as % of the dark control ± S.E. Demonstrates significant RL/FR reversibility of hypocotyl growth and cotyledon expansion in both wild type and *lh* mutant in etiolated seedlings, but only in the case of wild type in de-etiolated seedlings.

On the basis of physiological (Mancinelli, 1984; Mancinelli and Rabino, 1985), spectrophotometric (Heim *et al.*, 1981; Brockmann and Schäfer, 1982;

Jabben and Holmes, 1983) and immunological (Shimazaki *et al.*, 1983; Shimazaki and Pratt, 1985; Tokuhisha *et al.*, 1985) evidence, the existence of two pools of P have been proposed: *l*P, predominant in D-grown seedlings, and *s*P, predominant in light-grown seedlings. The observation of reduced growth of hypocotyls upon re-etiolation of the de-etiolated wild type compared to the *lh* mutant (the D-controls in Fig. 5.2) is consistent with the idea of *s*P functioning in the wild type but not in the *lh* mutant. Determination of the P content of both flower petals and Norflurazon-bleached leaves indicated that the P content of light-grown tissue of the *lh* mutant is about 35-50% of that in the wild type (Chapter 4). If this hypothesis is correct, the difference between mutant and wild type can be attributed to *s*P absent in the *lh* mutant. The detectable P in the *lh* mutant is proposed to be the steady state level of *l*P. An analysis of the mutant using antibodies which recognize *s*P would be desirable to verify the hypothesis. However, even if *s*P is present but fails to function, this *lh* mutant could provide an invaluable tool to answer the question, what the relative importance is of the *l*P and *s*P pools, and the BL-photoreceptor in light-grown plants.

Two experiments were designed in an attempt to ascribe a function of the P, deficient in the *lh* mutant, to a particular physiological response in WL-grown plants. Many species (Downs *et al.*, 1957), including cucumber (Gaba and Black, 1985a), respond by increased elongation growth to a short irradiation with FR at the end of the photoperiod in a WL/D cycle. This end-of-day FR effect was studied for hypocotyl elongation. Seedlings germinated in D were transferred to regimes consisting of 12 h WL/12 h D or 12 h WL/5 min FR/11 h 55 min D. Figure 5.5 demonstrates that the wild-type hypocotyls exhibit a significant stimulation of growth in length as a result of end-of-day FR. In contrast the *lh* mutant fails to respond. The lack of an end-of-day FR response is therefore ascribed to the P deficient in the *lh* mutant. If the hypothesis proposed here is correct this response of the hypocotyl to light is mediated by *s*P.

When one cotyledon is covered with aluminium foil and the plant is irradiated from above curvature towards the uncovered cotyledon occurs in WL and RL, but not in BL or D (Fig. 5.6). This response of de-etiolated seedlings due to RL absorption, presumably by P in the cotyledons, described earlier by Shuttleworth and Black (1977), is clearly absent in the case of the *lh* mutant. This suggests that this response in light-grown seedlings is an additional response regulated by *sP*.

While the precise nature of *s*P remains unknown, it may be a product of a gene different from that for *l*P or be a type of P resulting from post-translational modification of the *l*P in a particular cell compartment. The

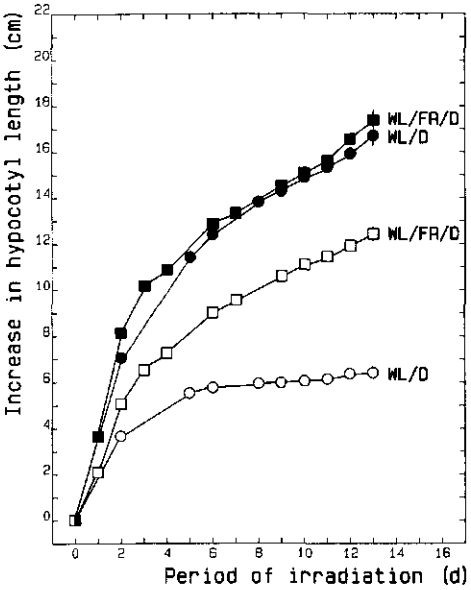

Fig. 5.5 End-of-day far-red (FR) response of hypocotyl growth of cucumber wild type (open symbols) and *lh* mutant (closed symbols) at 25 °C. Light treatment: 3 d darkness (D) followed by 15 d with 12 h white light (WL)/12 h D or 12 h WL/5 min FR/11 h 55 min D. Fluence rates: WL: 11.7 W m^{-2} [PAR, 54 μmol m^{-2} s^{-1}]; FR: 13.4 μmol m^{-2} s^{-1}. Demonstrates an end-of-day FR response in wild type which is absent in the *lh* mutant. For clarity only the S.E. for the last data points are shown, others were all < 5% of the mean.

lh mutant is not defective with respect to the *l*P and its action in etiolated plants, only with respect to the action of P in light-grown plants. However, these uncertainties do not limit the use of this mutant for understanding the function of these different phytochrome types. A mutant, the de-etiolated plants of which fail to respond to the RL region of the spectrum, will enable the relative roles played by BL in physiological processes to be studied unequivocally. As yet this has been impossible in long-term irradiation experiments.

The growth of the *lh* mutant and wild type under continuous BL of relatively low irradiance (Fig. 5.2; $\approx$ 4 μmol m^{-2} s^{-1}) suggested that they

are both inhibited by BL. A question therefore arises: why does the *lh* mutant grow long in WL containing a considerable BL component? In an attempt to answer this further long-term growth experiments have been carried out.

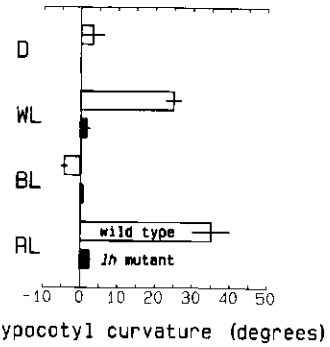

Fig. 5.6 Hypocotyl curvature of de-etiolated seedlings of wild type (pretreated with 30 h white light (WL [45 μmol m^{-2} s^{-1}]) and *lh* mutant (pretreated with 54 h white light (WL [45 μmol m^{-2} s^{-1}]), induced by wrapping one cotyledon with aluminium foil, in darkness (D); WL, 45 μmol m^{-2} s^{-1}; blue light (BL), 3.6 μmol m^{-2} s^{-1} or red light (RL), 3.6 μmol m^{-2} s^{-1} given from above for 24 h at 25 °C. Curvatures are expressed as degrees ± S.E. towards the uncovered cotyledon. Demonstrates curvature induced by WL and RL in the wild type and no response in the *lh* mutant.

Examination of the seedlings in growth experiments where inhibition in continuous BL has been observed (Fig. 5.2) reveals that at the end of the experiments the plants had only cotyledons (no 'true' leaves had developed). It is therefore possible to put forward the working hypothesis that the inhibition of growth by BL is temporal and occurs while the food reserves in the cotyledons are being utilized for growth and that the plants failed to develop long hypocotyls in these long-term growth experiments because the low BL fluence rates used could not sustain sufficient photosynthesis for growth. Penny *et al.* (1976) demonstrated that for cucumber, photosynthesis by the cotyledons was an absolute prerequisite for growth and development of true leaves. In the case of *Sinapis* (Beggs *et al.*, 1980) evidence for a loss of BL effectiveness for inhibition of hypocotyl growth during de-etiolation has been published. While little is known about the temporal pattern of activity of the different photoreceptors responsible for inhibition of hypocotyl growth in cucumber (Chapter 3), the evidence for the operation of a BL-photoreceptor, as well as phytochrome, is overwhelming (Attridge *et al.*, 1984; Cosgrove, 1981; Gaba and Black, 1984; Mohr, 1986).

To test this hypothesis 3-d old D-grown seedlings were first transferred to continuous BL. After 5 d, half of the plants were transferred to continuous WL containing the same fluence rate in the BL spectral region as in the continuous BL. Examination of Fig. 5.7 reveals that the *lh* mutant

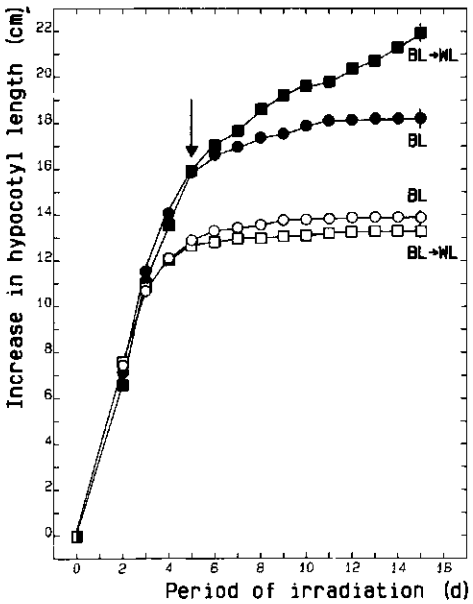

Fig. 5.7 Hypocotyl elongation in continuous blue light (BL; 0.8 W m^{-2} [3.0 μmol m^{-2} s^{-1}]) and upon transfer from BL to white light (WL; 7.0 W m^{-2} [PAR, 32 μmol m^{-2} s^{-1}]; containing 3 μmol m^{-2} s^{-1} BL) of cucumber wild type (open symbols) and *lh* mutant (closed symbols) at 25 °C. The arrow indicates the transfer to WL. Suggests that the growth of the hypocotyl of the *lh* mutant in continuous BL is restricted by photosynthesis. For clarity, only the S.E. for the last data points are shown, others were all <5% of the mean.

plants transferred from BL to WL grew longer than those maintained in BL. In addition, the wild type remained inhibited upon transfer to WL. This increase in growth of the *lh* mutant upon transfer to the WL is attributed to increased photosynthesis, enabling the growth of the hypocotyl where inhibition *via* phytochrome is absent. An indication of this is that at the end of the experiment the wild type and *lh* mutant had developed true leaves when transferred from BL to WL, whereas in continuous BL they had not.

To quantitatively confirm this result a more precise experiment was

designed in which two identical cabinets, enabling simultaneous irradiation of the plants with BL and RL, were used. Fig. 5.8 demonstrates that after the

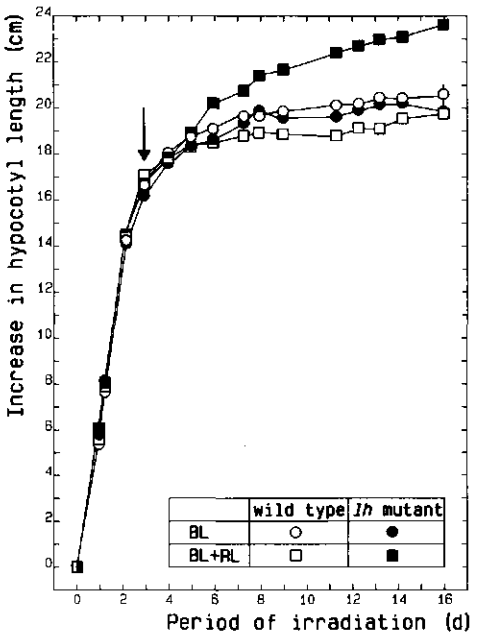

Fig. 5.8 Hypocotyl elongation in continuous blue light (BL; 2.3 μmol m^{-2} s^{-1}) with or without supplementary red light (RL; 13.2 μmol m^{-2} s^{-1}) of cucumber wild type (open symbols) and *lh* mutant (closed symbols) at 25 °C. The arrow indicates the onset of the RL. Confirms that photosynthesis limits the growth of the hypocotyl in continuous BL. For clarity only the SE for the last data points are shown, others were all <5% of the mean.

start of simultaneous irradiation with RL a significant increase in hypocotyl length results in the case of the *lh* mutant, but not in the wild type. At the end of the experiment both the wild type and *lh* mutant plants had significantly higher dry weights in simultaneous BL and RL than those maintained in continuous BL alone (Fig. 5.9). In addition, the wild type and *lh* mutant had a marked stimulation of the surface area of the true leaves. However, the expansion of cotyledon and true leaf surface area in the presence of RL in the wild type is very much greater than the *lh* mutant. The expansion of the cotyledons was previously demonstrated to be under P control (Fig. 5.4). Since expansion of the cotyledons is a prerequisite for

development of photosynthetic capacity, and photosynthesis is a prerequisite for further development of the seedling (Penny *et al*, 1976) it can be concluded that P action, if not directly, indirectly results in the

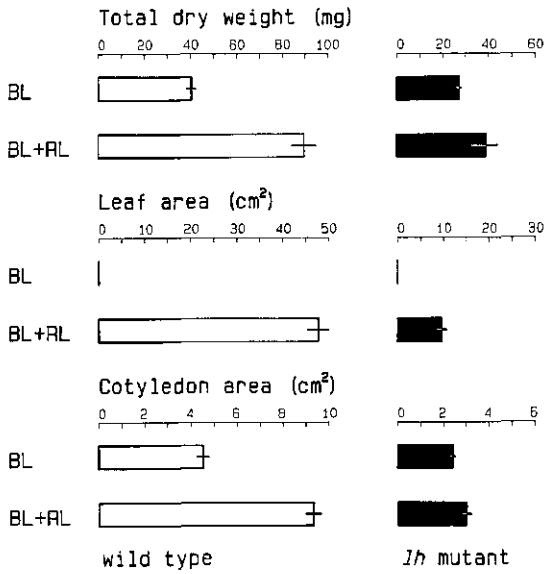

Fig. 5.9 The dry weights of plants and the area of cotyledons and true leaves ± S.E. at the termination of the experiment outlined in Fig. 5.8. Demonstrates that stimulation of leaf development occurs in the presence of supplementary red light (RL) and that the plants have a significantly higher dry weight than those maintained continuously in blue light (BL).

development of the leaves. In other words, the enhanced growth of leaves in the wild type is determined by two components: a strong P effect and a photosynthetic effect, whereas that of the *lh* mutant is predominantly determined by the photosynthetic effect. The paradoxical question why the *lh* mutant grows long in WL, is answered: the inhibition by the BL component in WL either stops or can be compensated by enhanced photosynthesis, resulting in enhanced elongation growth since inhibition by RL or WL working *via* P fails to function. The difference in growth of the hypocotyls in continuous BL between wild type and mutant observed in Figs. 5.7 and 5.8 can be accounted for on the basis of the BL fluence rate used. What is important here is the response to additional RL relative to the continuous BL control.

The long hypocotyl of the *lh* mutant is very similar to that of a wild

type plant treated with GA. A 'seed soak' of the wild type with 10^{-5} M GA_{4+7} resulted in wild-type seedlings after 6 d WL treatment with the same hypocotyl length as the untreated *lh* mutant of the same age (Fig. 5.10).

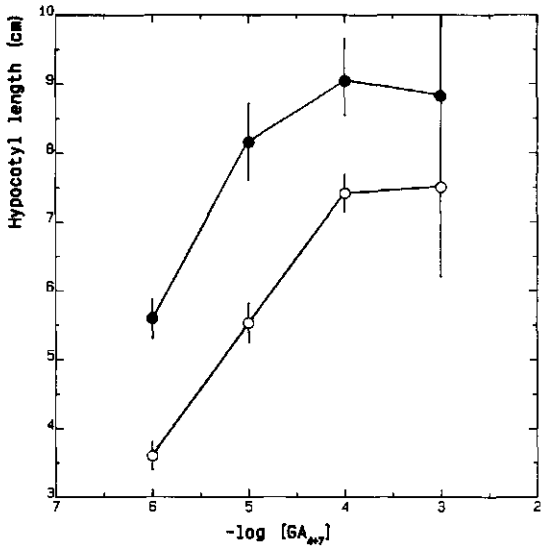

Fig. 5.10 Gibberellin (GA_{4+7}) dose-response curves of hypocotyl elongation of cucumber wild type (o) and *lh* mutant (•) after 5 d with a daily irradiation schedule of 8 h WL (8 W m^{-2} [PAR, 36 μmol m^{-2} s^{-1}] and 16 h darkness at 25 °C. Controls of wild type and *lh* mutant without GA_{4+7} were 32.5 and 56.2 mm, respectively. Demonstrates that the proportional response was similar in wild type and *lh* mutant.

There is also striking similarity of the behaviour of the *lh* mutant to the 'giant' *lv* pea mutant (Reid and Ross, 1988). Two possible ways in which a giant plant could arise by modification of GA metabolism are: an over-production of GA or an increase in sensitivity to GA. The levels of GA's have not yet been measured for the *lh* mutant. The proportional response for a given dose of GA_{4+7} was the same in wild type and *lh* mutant (Fig. 5.10). This indicates that the *lh* mutant was not saturated by endogenous GA. Furthermore, whereas the *lh* mutant, compared to the wild type, has long hypocotyls in WL and RL (Fig. 5.3), hypocotyl lengths of the *lh* mutant and the wild type are similar in BL or UV-A (Fig. 5.2). Differences in GA levels alone could not explain this observation. Inhibition of hypocotyl elongation due to irradiation decreased in wild type seedlings treated with exogenous GA (Fig. 5.11). However, this decrease was similar in RL and BL treated seedlings, indicating that the influence of GA was independent from the

effect of the light treatments. It therefore appears likely that the *lh* mutant is not a GA over-producer.

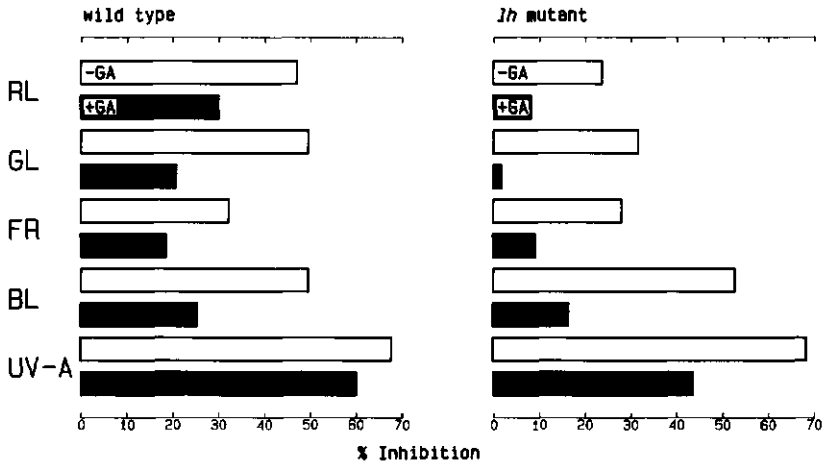

Fig. 5.11 Inhibition of hypocotyl elongation of cucumber wild type and *lh* mutant seedlings with and without treatment with 10^{-4} *M* gibberellin (GA_{4+7}; 'seed soak') in red (RL), green (GL), far-red (FR), blue (BL) or UV-A light (3.6 μmol m^{-2} s^{-1}), given as % of the apropriate dark-control. Demonstrates that the influence of GA_{4+7} is independent of the effect of the light treatments.

It cannot be excluded that the *hy-3* mutants of *Arabidopsis*, which contain IP (Koornneef *et al.*, 1980), show reduced levels of P in mature seeds (Cone, 1985) and physiological characteristics resembling P defiency, are of a similar type as the cucumber *lh* mutant.

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6. SHORT TERM GROWTH EXPERIMENTS

6.1 Introduction

Plants are capable of responding rapidly (within seconds or minutes) to changes in the light environment. In order to detect rapid responses in elongation growth, sensitive measuring methods have to be applied, since growth rate changes will be at most a few μm per second. While conventional growth studies using a ruler provide valuable information over longer periods, short-term changes will provide additional information. To obtain high-resolution measurements of elongation changes in seedlings of *Cucumis*, Meyer (1968) used a displacement transducer (linear variable displacement transducer), which enabled accurate continuous measurement of growth. Since that time displacement transducers have frequently been used to study rapid growth responses to light (e.g. Penny *et al.*, 1974; Gaba and Black, 1979; Loveys, 1979; Pike *et al.*, 1979; Morgan *et al.*, 1980; Cosgrove, 1981; Bleiss and Smith, 1985; Kristie and Jolliffe, 1986; García-Martínez *et al.*, 1987; Shinkle and Jones, 1988). Other, less frequently used techniques are capacitance auxanometry (Gordon and Dobra, 1972), time-lapse photography (Hart *et al.*, 1982; Baskin *et al.*, 1985) and video registration (Jaffe *et al.*, 1985; Yahalom *et al.*, 1987).

Time-lapse photography and video registration have a great advantage over other techniques: there is no direct contact with the plant. Infra-red light, which has no effect on growth, can be used for these observations. These techniques have often been used to study phototropic responses. The use of computer-assisted image analysis as described by Jaffe *et al.* (1985) enables fully automated data analysis. This is an improvement compared to the 'hand-controlled' tracing of pictures or video images, as it provides a much higher resolution.

The high-resolution growth measurements have disadvantages compared to conventional ruler measurements: only a small number of plants can be measured. Due to the variability in plant growth between individual seedlings and possible variations in experimental conditions during the individual measurements there is variability in the magnitude of the measured responses. This can be avoided by normalizing the growth in each experiment to growth under particular standard conditions, e.g. the growth rate in darkness (D), and by the use of computer-aided averaging of multiple

measurements of individual seedlings.

Several short-term growth studies have concerned so called 'transient' phenomena. Such transients, while not making any significant contribution to the total elongation, can provide information about existing differences in kinetics of several growth responses (Cosgrove, 1981; Gaba and Black, 1983b). The transients appear during the transition of one steady-state growth rate to another. They have been observed after transition from D to light or vice versa and after changing the fluence rate or light quality (e.g. Meyer, 1968; Cosgrove and Green, 1981; Cosgrove, 1982; Gaba and Black, 1983a; Child and Smith, 1987; Bleiss *et al.*, 1987). Transients in growth rate have also been observed after application of growth regulating substances, e.g. auxin (Green and Cummins, 1974; Pearce and Penny, 1986).

Inhibition mediated by the blue light (BL) photoreceptor may be distinguished from that *via* phytochrome (P) on the basis of the kinetics of growth inhibition (Chapter 3). This has been shown in experiments using short-term growth measurements. BL inhibits growth within 60 s, whereas the RL responses begin 15 to 90 min after the onset of irradiation, depending upon the species (Meyer, 1968; Cosgrove, 1981; 1982).

In the present study, high-resolution growth measurements have been used to compare the growth responses of the long-hypocotyl (*lh*) mutant of cucumber and its wild type. Preliminary studies were carried out manually using a horizontal microscope and a computer controlled growth meter was subsequently designed and constructed.

6.2 Material and methods

6.2.1 Plant material

The long hypocotyl (*lh*) cucumber mutant and its wild type (described in chapter 2) were used in the experiments.

6.2.2 Light sources

A description of the light sources used in these experiments can be found in appendix 'Light sources' and in 6.2.5. The fluence rates used in particular experiments are described in the figure legends.

6.2.3 Preliminary 'manual' growth measurements

Seeds of wild type and *lh* mutant were sown in Petri dishes on filter

paper moistened with distilled water and allowed to germinate in D at 25 °C. Seedlings were selected for study after 4 or 5 d. The hypocotyl growth of individual seedlings was monitored at 15 min intervals using a horizontal microscope with the seedling silhouetted against a green safelight during measurement. The D growth rate (periodic exposure to green safelight during measurement) was followed until stable. The seedlings were then irradiated from above with either narrow-band BL or RL. The growth was monitored over a 2.5 h period and the mean growth rate was calculated.

6.2.4 Continuous growth measurements

Seeds of wild type and *lh* mutant were sown in earthenware flower pots (dia. 12 cm) filled with a mixture of 50% vermiculite and 50% perlite (v/v) and saturated with water. After 4 d incubation in D at 25 °C 3 seedlings of similar height (≈ 4 cm) were selected and each individual seedling was carefully attached to the growth-measuring apparatus, manipulating the seedlings for ca. 1 min under a dim green safelight. The growth rate in D at 25 ± 0.3 °C was monitored for at least 4 h before the start of irradiation.

6.2.5 The apparatus for continuous growth measurement

A schematic diagram of the custom designed growth-measuring apparatus, constructed in the Laboratory for Plant Physiological Research, is shown in Fig. 6.1 and 6.2. The apparatus was designed by W.J.M. Tonk and R.M. Bouma and constructed from commercial and custom built components by J. v. Kreel in collaboration with other members of the technical staff of the laboratory. The measuring-device is mounted on a vibration-free table, consisting of a heavy steel plate which rests on air cushions (≈ 132 kPa) and from which a heavy (≈ 300 kg) base filled with grit is suspended.

Three separate devices are mounted on one table, each enclosed in an air- and light-tight, thermally isolated chamber. These chambers are mechanically isolated from the table and measuring-devices. Humidified air circulates through the three interconnected chambers and a climate-control unit controls the air temperature, humidity and flow rate. To avoid undesirable light reflections (and corrosion) all aluminum components are anodized or painted black. The complete apparatus is enclosed in a temperature-controlled room maintained at 25 ± 1 °C in D. A second set of three measuring units is under construction.

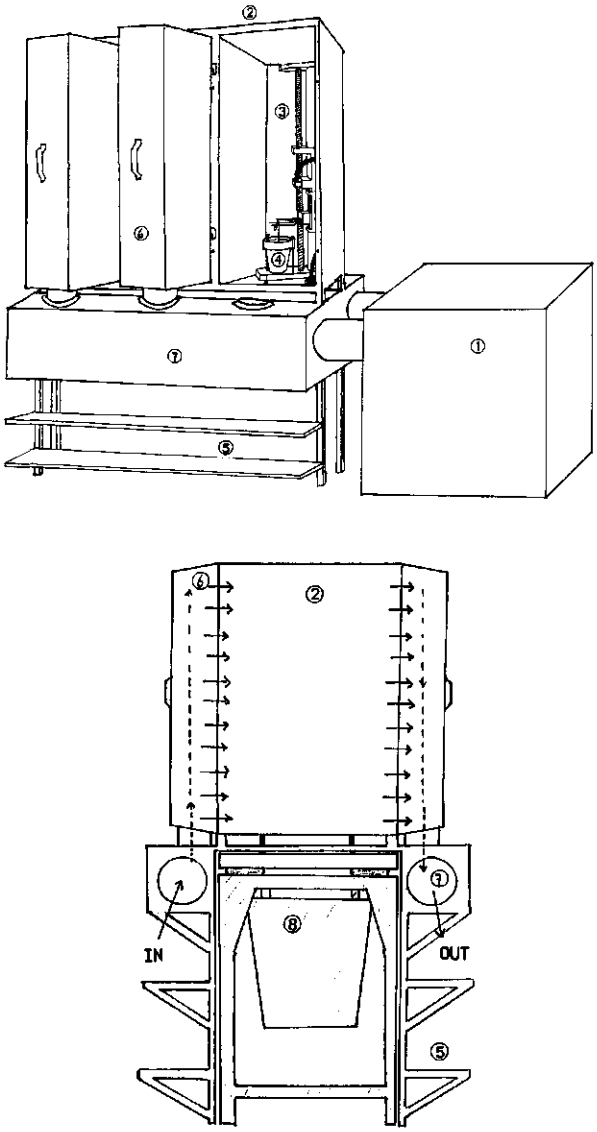

Fig. 6.1. A schematic diagram of the growth-measuring apparatus and the temperature-, humidity- and air flow control unit: front view without the vibration free table and with one chamber opened showing one measuring-device with a seedling attached to it (**above**); side view showing the vibration free table underneath the chambers and the air flow in and out of the chambers (**below**). (1) climate control unit; (2) chamber; (3) measuring device; (4) flower pot with seedling; (5) shelves for irradiation equipment and input and output devices; (6) hollow lid with special filter for a uniform air flow; (7) air flow chamber; (8) vibration free table.

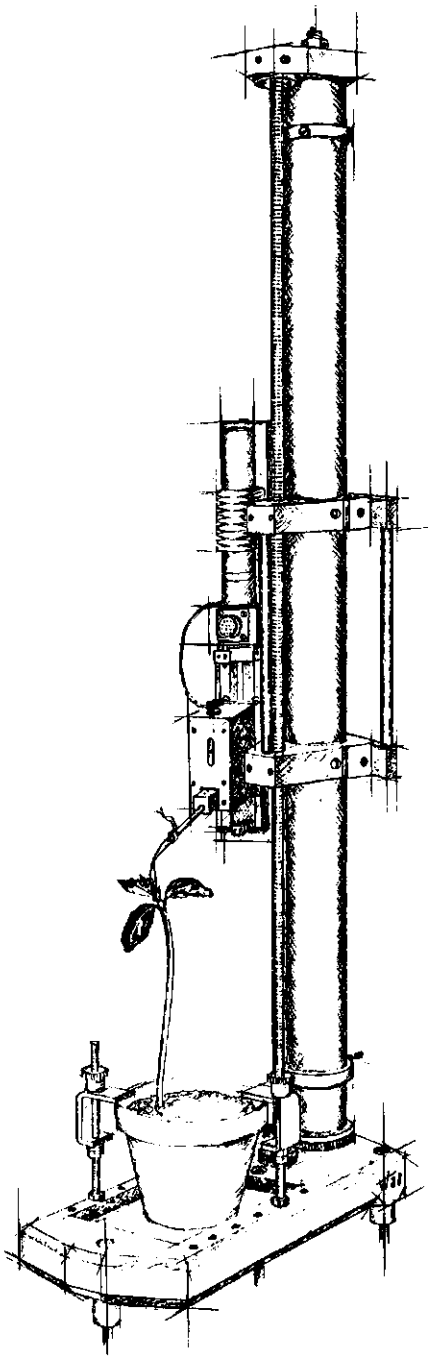

Fig. 6.2 A measuring device with a seedling (fibres for irradiation not shown). Detail (3) from Fig. 6.1.

A single board microprocessor (ARCOM, based on a ARC41, Z8 Basic/DEBUG computer with realtime clock/calendar for control, measurement and data logging, Tekelec Airtronic, Zoetermeer; Fig. 6.3) controls the whole system.

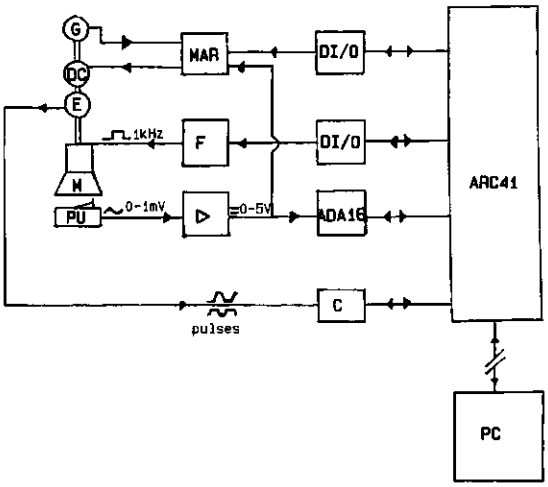

Fig. 6.3. A flow diagram of the translation unit of the growth-measuring apparatus. Measurement: (E) encoder; (C) counter. Steering: (G) tacho generator; (DC) motor; (MAR) adjusting amplifier; (F) function generator; (M) membrane; (PU) pick-up element; (DI/O) digital input/output; (ADA16) analog/digital converter; (▷) amplifier. For more detailed explanation see text.

A special program has been written for the microprocessor to enable selection of different parameters, e.g. cycle time and irradiation schedule. The cycle time is the time interval between two consecutive adjustments of the translation unit, i.e. the time between two growth measurements. The minimum cycle time is 1 s. The microprocessor is connected to a personal computer (PC) where further data-acquisition takes place.

A 'measuring-unit' (=translation unit) is mounted on a column with a motorized spindle (M12x3) and can be raised or lowered to position it at the actual height of the plant (range ≈ 70 cm). It is possible to place two translation units on one column to enable differential measurement. The pot containing the seedling is clamped to the base of the column. The seedling is attached to the translation unit by ligature thread impregnated with silicon rubber to avoid abrasive damage to the stem. In the experiments described here the thread was looped around the hypocotyl hook.

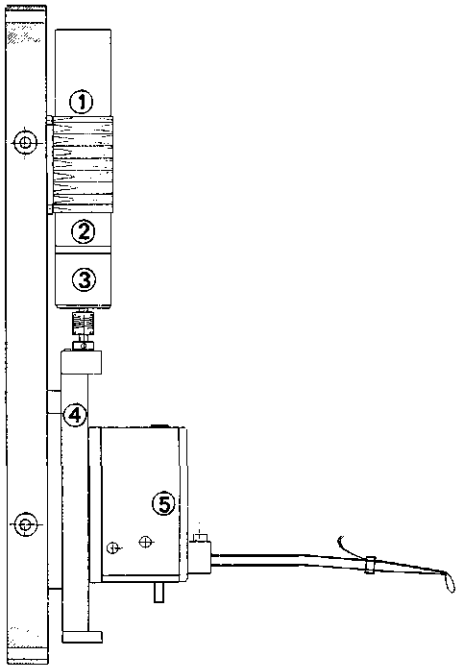

Fig. 6.4. The translation unit of the growth-measuring apparatus. (1) DC motor with tacho generator and amplifier; (2) satellite reduction gear; (3) optic incremental encoder; (4) sledge mounted on the spindle; (5) floating zero. For more detailed explanation see text.

The translation unit (Fig. 6.4) consists of an aluminium base plate with the following devices attached to it:

1. A cylindrical coil DC motor (Faulhaber) with tacho generator (Mattke ARIB) combined with a four quadrant, linear adjusting amplifier MAR 9/03.
2. A satellite reduction gear (1:10), type UE 30 CC (Faulhaber).
3. Incremental encoder (type UT 100 CC of Micro Control). This encoder counts the number of rotations of a selected angle of the motor. For the experiments presented a setting of 500 points per complete revolution has been used. However, it is possible to use 1000 points per revolution to improve resolution.
4. A sledge, mounted by high quality roller bearings (Föhrenback, type RS 040-130-050) on a precision spindle (M5x0.5).
5. The 'floating zero' is mounted on the sledge (Fig. 6.4). This component of the translation unit enables repeated indication of a preselected

electrical signal (equivalent to a position within a few nm) each time a measurement is completed.

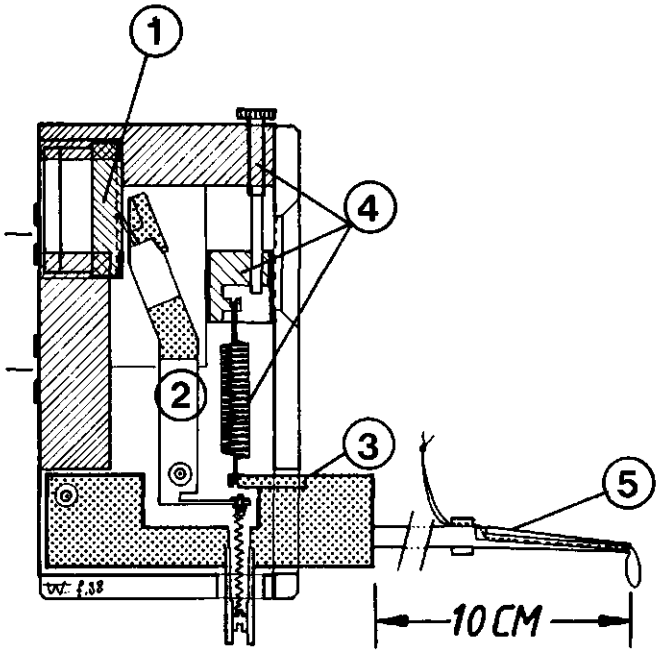

Fig. 6.5. Detailed diagram of the 'floating zero'. (1) microphone; (2) pick-up element with arm; (3) measuring-arm block; (4) calibrated pull spring; (5) measuring arm. For more detailed explanation see text.

The floating zero, in Fig. 6.4 (5), is illustrated in detail in Fig. 6.5 and consists of the following components:

1. A microphone (A.K.G., type D58E) modified with a Melinex I.C.I. 75 μm membrane and iron assembly cap.
2. A pick-up element (B&O, type MMC-4) with a part of the tangential arm (3152378) and a heavy-duty clock balance shaft mounted in sapphire bearings.
3. Measuring-arm block (aluminium 52 ST) coupled to the pick-up arm with an adjustable gas-reduction spring from a gaslighter (BIC-B3). The shaded block with the measuring arm is held in position by means of the balance shaft/ sapphire combination (2).

4. A calibrated stainless steel pull spring (Tevema-Barnes, type T-41030, 50-300 g linear adjustable), used to adjust the upward tension on the plant.
5. The measuring arm with a loop of thread.

The measuring arm from the floating zero is connected to the plant, *via* the hollow tip, with an adjustable loop of thread. A constant upward tension is maintained on the plant. Within the floating zero, this tension can be regulated with an adjustable spring (4) from 1 to 20 g. For the experiments described the upward tension was 1.2 g. A very small upward tension is possible when bearings, transmissions and the pick-up signal amplifier of the floating zero are tuned optimally. Each translation unit can be tuned separately. This is necessary because of differences in the individual components.

The sledge with the floating zero is mounted on a precision spindle with an adjustment range of 10 cm. A DC motor raises or lowers the sledge. The measuring arm is coupled, *via* a transmission, to the pick-up element. The function generator induces a 1 KHz vibration of the membrane. The pick-up needle touches the membrane and converts the vibration into an electrical signal between 0 and 1 mV (Fig. 6.3). This AC signal is amplified and converted to 0 - 5 V DC and then converted to a digital signal. The ARCOM microprocessor follows each translation unit in sequence, activating the function generator and checking the level of the signal from the floating zero. When the pre-set cycle time is reached and the signal is above a certain pre-selected threshold value, the microprocessor 'orders' the motor to move upwards. The measuring arm is then raised with the sledge. Due to the loop of thread attached to the plant and the spring that adjusts the upward tension, the measuring arm tilts. The sapphire bearings, the balance shaft and a friction-free transmission ensures that when the measuring arm moves upwards the pick-up needle moves backwards. The tension of the needle on the membrane reduces and the induced signal decreases. When this signal decreases beneath a threshold value the motor stops. In the experiments presented a threshold value of 30 mV was used. Simultaneously the encoder counts the number of rotation angles of the motor on the spindle (Fig. 6.4 [3]). Inside the encoder are two metal discs with 500 equally spaced apertures: one is stationary and one rotates simultaneously with the motor. Two LED's are positioned on one side of the discs and two photodiodes on the other. The light from the LED's is transmitted only when the apertures in

both discs are lined up; during a complete revolution there will be 500 alternating light and D periods. The apertures of the two photodiodes are positioned so that a light period on one detector corresponds to a dark period on the other. Both photodiodes produce a signal each time a light period has been detected. This results in two square wave signals from the encoder to the counter, which are 90° out of phase. Direction of rotation, and as a consequence the direction of the motor-movement, is determined by observing which of the signals is the leading waveform. The information concerning the extent and direction of the movement is relayed to the microprocessor, where it is stored in a special memory. If the adjustment can not be completed within one cycle time a special code is stored, indicating this event, and the adjustment continues in the next cycle. The total value, corresponding to the growth over the number of cycle times, is subsequently stored at the cycle time at which the adjustment is completed.

Depending upon the desired precision, a threshold value of 20 to 100 mV is set at which the motor stops. The pick-up signal determines (*via* the adjusting amplifier and the reduction gear) the velocity of the motor following growth of the plant. Decrease of the signal causes the motor to slow down. When this velocity is too low the motor 'sticks'. This causes the processor to keep 'trying' to adjust the measuring unit involved. When the end velocity is too high an 'overshoot' occurs; the motor does not stop in time. These effects result in a precision which is out of the required ìm range. These problems are caused by the satellite reduction gear of 1:10. This value was estimated as appropriate during the development of the apparatus, but appears to be too low. With a reduction gear of 1:20 a higher precision will be possible.

In the experiment described the hypocotyl was irradiated just beneath the hypocotyl hook. For this purpose, a two-armed optical fibre (1600 mm long, 4 mm dia; Schott Nederland B.V.) in association with a projector assembly fitted with a 250 W quartz-iodide projection lamp was used. The projector was equipped with a heat-filter and an interference filter (Balzer B40 type, Balzer Liechtenstein), 658 nm or 459 nm, 10 nm half-band width at 50% of the transmission maximum. The fibres were held in position by adjustable clamps attached to special arms on the translation unit (not shown in the figures). In this way the fibres follow the growing plant. It is also possible to irradiate from 4 directions with additional fibres. Furthermore, facilities for irradiation from above with white light (WL) for photosynthesis are under construction. The irradiation schedule is

programmed in the microprocessor, which automatically switches the lights on and off after a preselected number of cycle times and registers this together with the other data gathered at that specific cycle time.

The data stored in the buffer memory of the microprocessor are collected, decoded and analyzed with a PC situated in a separate room. Using a data-acquisition program written for the apparatus, it is possible to normalize and smooth each individual measurement, average a large number of measurements, calculate growth rates and lag periods with a resolution of 1 s and 1 μm. Calculation of the growth rate of a part of the plant is also possible using differential measurement with two translation units on one column. The results can be stored and displayed on terminal, printer or plotter. The data are stored in a way that enables the use of several commercially available data processing programs (e.g. Lotus 1-2-3).

6.2.6 Data manipulation and presentation of results

The increase in length was measured every 30 s in D, RL and WL, and every 10 s in BL (=cycle time). The mean growth rates (c.f. Table 6.2) are calculated using the individual growth rates from 1 h before onset of irradiation, and the first and third (BL) or fourth (RL and WL) hour after onset of irradiation. The lag period was determined as follows. At first the approximate time at which the inhibition started was estimated from the figure. The mean growth rate in the hour before this point, usually equal to the growth rate in D, was determined. The lag period was calculated by determining the time between the onset of the irradiation and the last point (=cycle time) at which the growth rate was higher than or equal to the mean growth rate. The mean lag period has been calculated as suggested by Rich and Smith (1986): the time between induction and first expression of the response is determined for each individual replicate, and then averaged to give a mean lag period for the population.

To enable detection of growth patterns below the noise level the data were smoothed where necessary. This was done by repeated application of a so called '3-point running mean'. This treatment of the data reduces the noise, but also the time-resolution. However, in experiments with a cycle time of 1 s the resolution after applying 10 times a 3-point running mean is still $\approx$ 10 s. To prevent of loss of time resolution, smoothing was not performed on the data from which the lag periods were calculated.

6.3 Results and discussion

The growth of the *lh* mutant and wild type under continuous BL irradiation (Chapter 5) suggested that they are both inhibited by BL, whereas the response to RL was severely reduced in the *lh* mutant compared to wild type. The growth rates of plants both in D and upon transfer to continuous BL and RL was monitored at 15 min intervals with the aid of a horizontal microscope (Table 6.1). In contrast to plants removed from absolute D, which showed no difference in absolute hypocotyl growth (Chapter 5), wild-type dark-controls in this preliminary experiment exhibited a small, but significantly lower growth rate than the *lh* mutant. It is proposed that this is a response of the wild type, *via* light-stable P (*s*P), to the repetitive exposures to green safelight. Despite the limitations of this technique it is clear from the results that the growth rate of hypocotyls is significantly reduced during the 2.5 h BL irradiation period in both the wild type and *lh* mutant. In contrast, RL is considerably more effective in wild type than in the mutant in bringing about a reduction in growth rate. These results provide additional evidence to that already presented in Chapter 5 that BL can inhibit hypocotyl growth in the *lh* mutant as well as in the wild type.

Table 6.1. Measurements of the mean hypocotyl growth rates ± S.E. (number of plants measured) of 4-5 d old dark-grown *lh* mutant and wild-type cucumber seedlings maintained in darkness (D) and over a 2.5 h period after transfer to continuous blue (BL) or red (RL) light (both ≈ 15 μmol m^{-2} s^{-1}) at 25 °C, measured with a horizontal microscope under green safelight.

	Wild type		*lh* mutant	
Treatment	Growth rate (mm h^{-1})	% D	Growth rate (mm h^{-1})	% D
D	1.38 ± 0.08(26)	100	1.60 ± 0.09(28)	100
BL	0.73 ± 0.07(8)	53	1.04 ± 0.12(10)	65
RL	0.65 ± 0.09(10)	47	1.34 ± 0.13(11)	84

To enable a comparison of short-term growth kinetics of wild type and *lh* mutant their growth responses were monitored using an apparatus for continuous growth measurement. Fig. 6.6 and 6.7 show the growth kinetics of an individual seedling of wild type and *lh* mutant in D and during BL irradiation, respectively. Growth curves of other individual seedlings were

qualitatively similar. Mean values are given in Table 6.2. These results confirm the preliminary observations that both wild type and *lh* mutant are inhibited by BL. The first inhibition appears to be temporary and occurs with a mean lag period < 2 min (Table 6.3). The individual lag periods measured were from a few seconds to a few minutes. After the initial inhibition there is some recovery, but the growth rate in BL remains lower than in D, indicating again that BL inhibits hypocotyl growth in both wild type and *lh* mutant.

Table 6.2. Measurements of the mean hypocotyl growth rates ± S.E. (number of plants measured) of 4-5 d old dark-grown *lh* mutant and wild-type cucumber seedlings maintained in darkness (D) and over the first and the third (in blue light (BL)) or fourth (in red light (RL) and in white light (WL)) hour after onset of irradiation. Measured with the growth-measuring apparatus. Examples of individual growth curves and fluence rates used are given in Fig. 6.6 to 6.11.

Wild type

	First hour		Third or fourth hour	
Treatment	Growth rate (mm h^{-1})	% D	Growth rate (mm h^{-1})	% D
D	1.41 ± 0.05(55)	100 ± 4		
BL	0.68 ± 0.04(16)	58 ± 4	0.88 ± 0.03	77 ± 7
RL	1.73 ± 0.09(18)	129 ± 7	1.19 ± 0.06	89 ± 7
WL	0.41 ± 0.03(12)	27 ± 2	0.42 ± 0.04	29 ± 4

lh mutant

	First hour		Third or fourth hour	
Treatment	Growth rate (mm h^{-1})	% D	Growth rate (mm h^{-1})	% D
D	1.28 ± 0.05(51)	100 ± 4		
BL	0.66 ± 0.05(17)	54 ± 4	0.96 ± 0.06	82 ± 6
RL	1.50 ± 0.06(18)	128 ± 8	1.22 ± 0.04	104 ± 5
WL	0.47 ± 0.08(12)	34 ± 4	0.44 ± 0.06	32 ± 4

Growth of etiolated seedlings of wild type and *lh* mutant in RL is shown in Fig. 6.8 and 6.9, respectively. Hypocotyl elongation was inhibited by RL in wild type, but not significantly in the *lh* mutant. However, the first

hour in RL a growth promotion was observed in both wild type and *lh* mutant. This promotion has not been observed in the experiment with the horizontal microscope. An important difference between the experiments was the geometry of irradiation. In the experiment with the microscope the seedlings were irradiated from above, directly on the cotyledons, while in the growth-measuring apparatus only a small part of the hypocotyl was irradiated. This could explain the different responses observed and it could explain why growth promotion by RL has not previously been reported. In addition, in the growth-measuring apparatus the transition was from a period of several hours absolute D to RL, whereas in the microscope experiments the plants received repetitive exposures to green safelight while the dark-growth rate was being determined.

The mean lag periods of wild type and *lh* mutant observed with the growth-measuring apparatus are longer than those observed with the horizontal microscope and those observed by Meyer (1968) and Cosgrove (1981). This could also be a result of the different location of irradiation. The influence of RL is predominantly mediated through light absorption by the cotyledons (Black and Shuttleworth, 1974; Cosgrove, 1981). When only the hypocotyl is irradiated the cotyledons possibly receive light transmitted through the hypocotyl. This could cause a delay and a decrease of the response.

In WL (Fig. 6.10 and 6.11) an initial inhibition occurs within minutes after the onset of the irradiation and a second inhibition a few hours later. This is likely to be due to a temporal separation of the effects due to the BL-photoreceptor and P as described by Gaba and Black (1979; Chapter 3).

Table 6.3 Mean lag periods (min) between the onset of the irradiation and the first detectable response of cucumber wild type and *lh* mutant seedlings. Measured with the growth-measuring apparatus.

Treatment	Wild type	*lh* mutant
BL	1.5 ± 0.5	1.4 ± 0.5
RL	147.6 ± 10.3	162.8 ± 13.3
WL	1.2 ± 0.6	0.6 ± 0.6

In etiolated seedlings no large differences are observed in growth responses

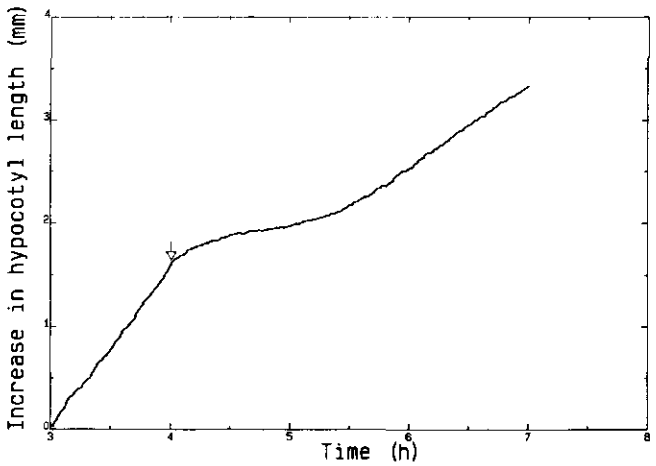

Fig. 6.6. The effect of blue light (BL; 34 μmol m^{-2} s^{-1} incident at both sides of the hypocotyl) on the hypocotyl elongation of an etiolated seedling of cucumber wild type. The arrow indicates the onset of BL. Attachment of the seedling to the apparatus at time 0; cycle time 10 s.

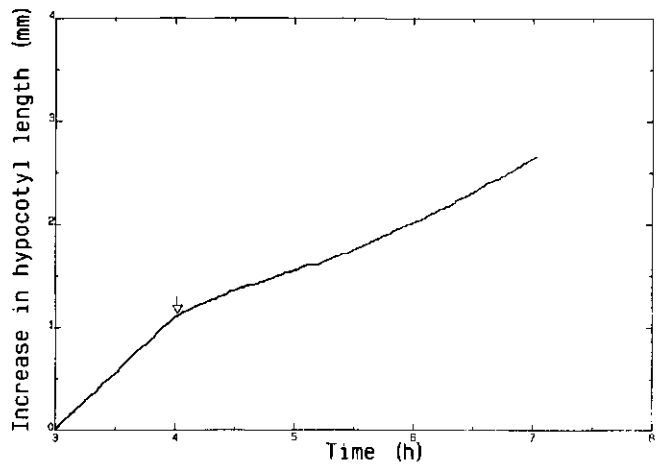

Fig. 6.7. The effect of blue light (BL; 34 μmol m^{-2} s^{-1} incident at both sides of the hypocotyl) on the hypocotyl elongation of an etiolated seedling of cucumber *lh* mutant. Otherwise as Fig. 6.6.

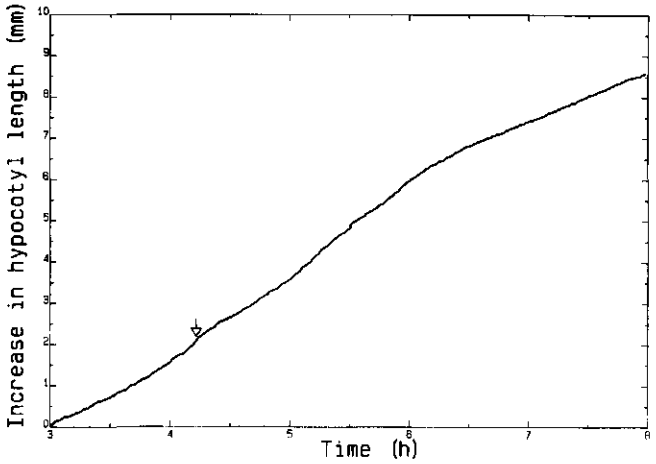

Fig. 6.8. The effect of red light (RL; 17 μmol m^{-2} s^{-1} incident at both sides of the hypocotyl) on the hypocotyl elongation of an etiolated seedling of cucumber wild type. The arrow indicates the onset of RL. Attachment of the seedling to the apparatus at time 0; cycle time 30 s.

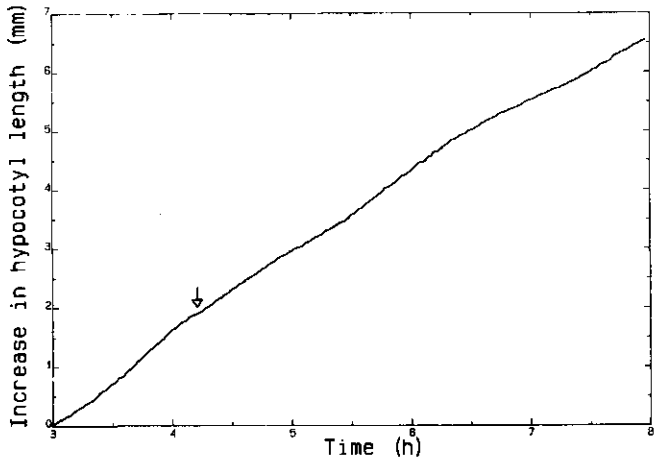

Fig. 6.9. The effect of red light (RL; 17 μmol m^{-2} s^{-1} incident at both sides of the hypocotyl) on the hypocotyl elongation of an etiolated seedling of cucumber *lh* mutant. Otherwise as Fig. 6.8.

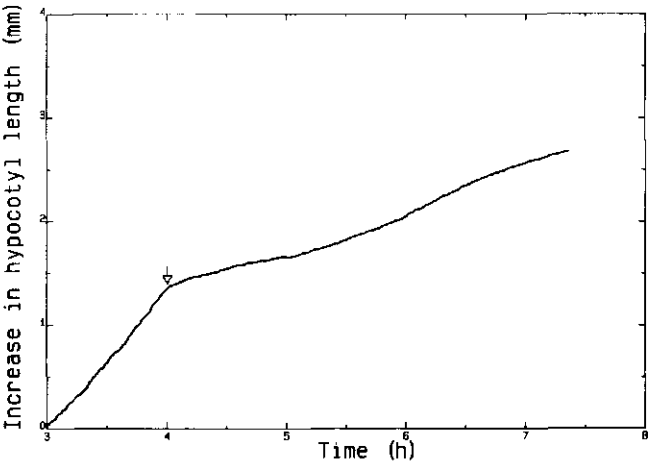

Fig. 6.10. The effect of white incandescent light (WL; 15 mW m^{-2} incident at both sides of the hypocotyl) on the hypocotyl elongation of an etiolated seedling of cucumber wild type. The arrow indicates the onset of WL. Attachment of the seedling to the apparatus at time 0; cycle time 30 s.

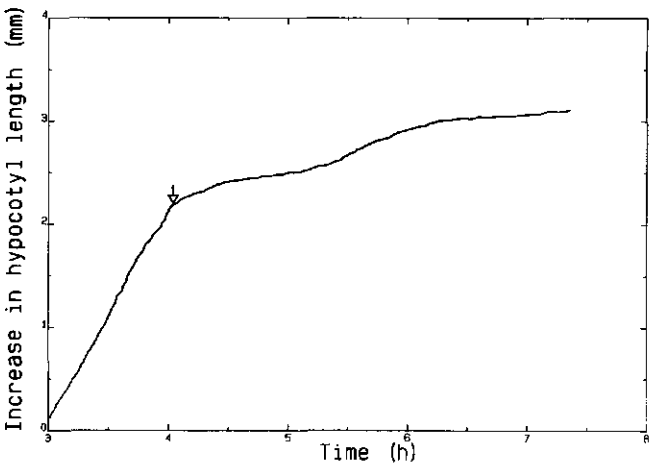

Fig. 6.11. The effect of white incandescent light (WL; 15 mW m^{-2} incident at both sides of the hypocotyl) on the hypocotyl elongation of an etiolated seedling of cucumber *lh* mutant. Otherwise as Fig. 6.10.

of wild type and *lh* mutant. This is consistent with the results from long-term growth experiments (Chapter 5). In chapter 4 it was proposed that the *lh* mutant lacks *s*P. In etiolated seedlings the bulk P pool consists of labile P (*l*P) and no differences were observed between P levels in etiolated seedlings of wild type and *lh* mutant. This could explain why there is only a small difference between the responses of etiolated seedlings. It is expected that short-term growth responses of de-etiolated seedlings of the wild type and the *lh* mutant will show larger differences, since there *the s*P present in the wild type is expected to play an important role in the control of elongation growth. Larger differences have indeed been observed in long-term growth experiments (Chapter 5).

The present experiments are the first carried out with this growth measuring apparatus. After further improvement of the equipment, e.g. with respect to resolution, it will be possible to study responses within a few seconds, such as transient growth rate overshoots (Cosgrove, 1982; Gaba and Black, 1983a; 1983b; Morgan and Smith, 1978) and growth oscillations (Kristie and Jolliffe, 1986). Fig. 6.12 shows the preliminary results from an experiment

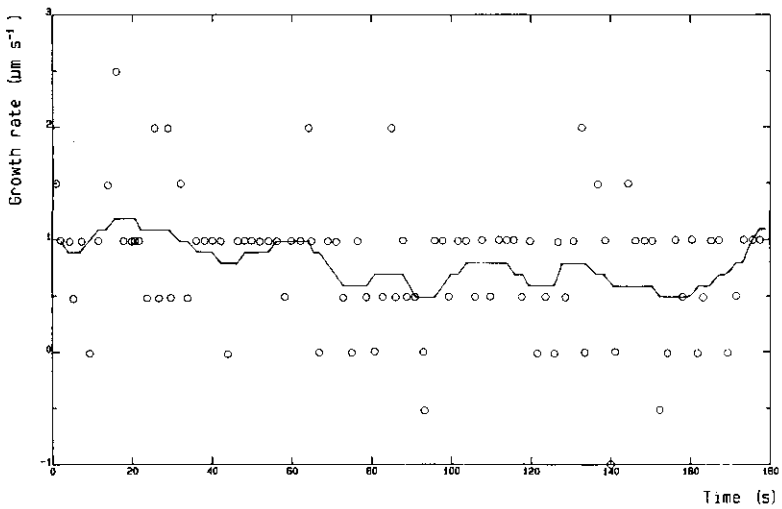

Fig. 6.12. High-resolution growth measurements of a cucumber wild type seedling in darkness at 25 °C. The growth was determined with a cycle time of 1 s.
a. Raw data.
b. Smoothed data (10 x smoothed with a 3-point running mean).

using a cycle time of 1 s. Smoothing of the curve certainly is required for conclusions about the occurrence of oscillations in growth rate. Although this procedure reduces the time-resolution it is still possible to measure these oscillations with a time resolution of 10 s and a growth rate resolution of 1 $\mu m\ s^{-1}$.

The equipment is highly suitable for further experiments with seedlings and full-grown plants. In these experiments the full potential of the equipment will be utilized. This will enable the measurement of growth responses within a few seconds or the monitoring of growth for several days under temperature-, humidity- and air flow-controlled conditions, with fully automated irradiation schedules and data-acquisition.

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7. LONG TERM GROWTH EXPERIMENTS WITH TOMATO

7.1 Introduction

In search for non-germinating, gibberellin (GA) responsive mutants, Van der Veen and Bosma (Koornneef *et al.*, 1981) isolated a non-germinating, GA-responsive tomato mutant, W616, characterized by an elongated hypocotyl and yellow-green leaves. Complementation tests with earlier described long hypocotyl mutants of tomato and linkage tests revealed that W616 was allelic with *aurea* (*au*) mutants. The *au* mutant resembled previously described *Arabidopsis* mutants characterized by an elongated hypocotyl when grown in white light (WL), pale green leaves, reduced germination, increased apical dominance and the absence of spectrophotometrically detectable phytochrome (P) (Koornneef *et al.*, 1980; Spruit *et al.*, 1980; Cone and Kendrick, 1985). Koornneef *et al.* (1985) showed that this *au* mutant of tomato, in contrast to its wild type, showed little or no effect of light on seed germination, anthocyanin synthesis and hypocotyl elongation. In addition the chlorophyll content is reduced, the chlorophyll *a/b* ratio is increased and the stacking of the thylakoids in the chloroplast is greatly reduced. Spectrophotometrically detectable P is absent or strongly reduced in its seeds, dark-grown hypocotyls, light-grown leaves, and roots. Spectrophotometric (Chapter 4) and immunochemical (Parks *et al.*, 1987) measurements have shown that etiolated tissue of the *au* mutant has a P content < 5% of the wild type. Spectrophotometric measurements of P in flower petals and Norflurazon-bleached leaves indicate that light-grown tissues contain 30-50% of the P level in the wild type (Chapter 4). The P detectable in light-grown plants of the *au* mutant is proposed to be light-stable P (*sP*) and the difference between P levels in mutant and wild type represents the steady state level of labile P (*lP*) in WL. The difference between responses of *au* mutant and wild type must therefore reflect the quantitative role of the P deficient in the *au* mutant. In this chapter the results of experiments concerning elongation growth of the *au* mutant and its isogenic wild type are presented and the possible role of the different P types in several elongation growth responses is discussed.

7.2 Materials and methods

7.2.1 Plant material

The long hypocotyl (*au* mutant of the tomato cv. Moneymaker and its isogenic wild type used in the present experiments have been described in Chapter 2. Apart from its long hypocotyls, the *au* phenotype is characterized by reduced seed germination, reduced chlorophyll content and reduced anthocyanin levels (Koornneef *et al.*, 1985).

7.2.2 Light sources.

A description of the light sources used in these experiments can be found in appendix 'Light Sources'. The fluence rates used are indicated in the figure legends.

7.2.3 Germination and dark incubation

Dark germination of the *au* mutant was low and retarded compared to the wild type (Koornneef *et al.*, 1985). However, pricking each seed with a sterile needle considerably increased the percentage of dark-germination of the *au* mutant. This technique was used for both the *au* mutant and the wild-type seeds. To compensate for the retardation of germination of the *au* mutant, seeds were sown 24 h before those of the wild type. Seeds of wild type and *au* mutant were surface sterilised for 5 min in a 1% (v/v) dilution of a Na-hypochlorite solution (commercial bleach), washed for 30 min in running tap water, pricked, sown in transparent plastic boxes (10x10x8 cm) on 11 layers of filter paper saturated with distilled water, and placed in darkness (D) for germination at 25 °C.

7.2.4 Screening under broad spectral band light sources

After 3 d (wild type) or 4 d (*au* mutant) incubation in D 10 seedlings were selected per box with uniform hypocotyl length, 4 boxes being used for each treatment. The seedlings were transferred to continuous UV-A, blue (BL), green (GL), red (RL), and far-red (FR) light of 2.1 μmol m^{-2} s^{-1} $\pm$ 5%. The dark controls were kept in wooden boxes covered with black polythene and placed in each of the light cabinets. The hypocotyls were measured daily under a dim green safelight, using a ruler.

7.2.5 Fluence-rate response to BL and UV-A

After 5 d (wild type) or 6 d (*au* mutant) incubation in D, 12 seedlings

were selected per box with uniform hypocotyl length, 4 boxes being used for each treatment. The seedlings were transferred to continuous UV-A or BL of different fluence rates. The dark controls were kept in wooden boxes covered with black polythene and placed in each of the light cabinets. Hypocotyl lengths were measured before (under a dim green safelight) and after the 24 h irradiation period, using a ruler. The results are presented as % inhibition compared to the dark control.

7.2.6 End-of-day far-red experiments

Seeds of wild type and *au* mutant were sown in earthenware seed pans and after 14 d were transplanted into 10 cm diameter plastic flower pots filled with potting compost. After a further 14 d at 23 °C in daily irradiation schedules of 14 h WL (35 W m^{-2} [PAR, 160 μmol m^{-2} s^{-1}]) and 10 h D plants were selected for uniform height and submitted to daily supplementary light treatments. After the daily WL period the following irradiation schedules were given: D; 20 min FR (20 μmol m^{-2} s^{-1}); 20 min FR followed immediately by 10 min WL. After 5 cycles the plants were returned to 14 h WL/ 10 h D for two cycles and then the total length of the internodes was measured. The increase in height of the plants ± S.E. is expressed as a percentage of the initial height of the plants on the day they received the first end-of-day FR treatment.

7.2.7 Simulated phototropism

Seedlings were grown in daily irradiation schedules of 16 h WL and 8 h D at 20 °C in earthenware seed pans (25x25x6 cm) filled with potting compost. After 8 d the seedlings were selected for uniform hypocotyl length and one cotyledon of each seedling was covered with aluminium foil. The seedlings were placed in continuous WL (11.7 W m^{-2} [PAR, 53 μmol m^{-2} s^{-1}) at 23 °C and irradiated from above. After 24 h each seedling was removed from the compost and photocopied. The angle of curvature of each hypocotyl was estimated from the photocopies using a protractor.

7.2.8 Presentation of results

Results are expressed as mean ± S.E. of representative experiments and have all been repeated at least once with qualitatively similar results. In some experiments the least significant difference (LSD) at the 5% level of probability is given, calculated according to the method of Tukey (1977).

7.3 Results and discussion

7.3.1 Screening under broad spectral band light sources

When germinated in D and transferred to continuous irradiation with broad band light sources of equal photon fluence rate for 4 d, both the wild type and the *au* mutant showed similar hypocotyl growth in D and FR (Fig. 7.1). In RL and GL, in contrast to the wild type, no significant inhibition

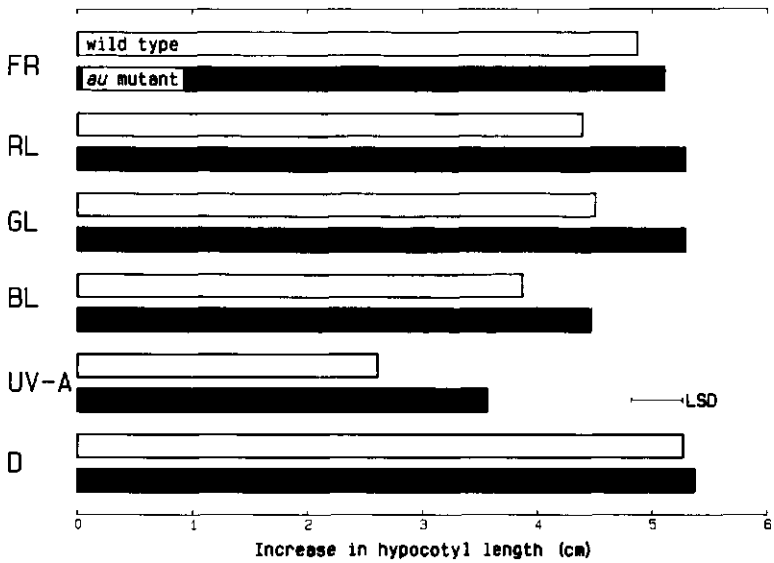

Fig. 7.1 The increase in hypocotyl length of the *au* mutant of tomato and its isogenic wild type grown for 4 d in broad band light (2.1 μmol m^{-2} s^{-1} ± 5%) at 25 °C; far-red light (FR), red light (RL), green light (GL), blue light (BL), UV-A or darkness (D). Seedlings were grown for 3 d (wild type) or 4 d (*au* mutant) in D before transfer to the light. The bar indicates the least significant difference (LSD) at the 5% level between the dark control and the light treatments.

was observed in seedlings of the *au* mutant. In Chapter 4 it was demonstrated that etiolated seedlings of the *au* mutant have no spectrophotometrically or immunochemically detectable *1*P. This deficiency of *1*P results in a loss of P control of hypocotyl elongation in etiolated seedlings of the *au* mutant. In BL and UV-A both wild type and *au* mutant were significantly inhibited compared to the dark controls, but a smaller response was exhibited in the case of the *au* mutant. Apparently inhibition of hypocotyl elongation due to

a BL/UV-A photoreceptor is retained in the *au* mutant, despite the lack of *l*P. This suggests that BL inhibition of elongation growth does not require the presence of Pfr or it is dependent on an extremely sensitive process regulated by the low level of Pfr present. To futher evaluate the role of the P deficiency in the *au* mutant on the inhibition of hypocotyl elongation growth by BL and UV-A, fluence-rate response curves were determined.

7.3.2 Fluence-rate response to BL and UV-A

Fluence-rate response curves for growth inhibition of etiolated hypocotyls occurring during a 24 h period in BL and UV-A show a clear difference in response range for wild type and *au* mutant (Fig. 7.2). The *au* mutant requires a higher fluence rate to be inhibited to the same extent as the wild type. The fluence rate of light sources used were probably not high enough for the *au* mutant to reach the maximum inhibition as measured in the wild type. In both BL and UV-A the curve for the *au* mutant is shifted approximately one order of magnitude to higher fluence rates. This difference in inhibition by BL and UV-A between the *au* mutant and its wild type represents a quantitative estimate of the role played by BL and UV-A absorption by *l*P in the photo-inhibition of hypocotyl growth. The inhibition at higher fluence rates in the *au* mutant could represent the BL/UV-A photoreceptor working alone, or in co-action with any 'residual' P, either *l*P or *s*P below detection limits.

7.3.3 End-of-day far-red experiments

Elongation growth of many species (Downs *et al.*, 1957), including tomato (Selman and Ahmed, 1962; Tucker, 1975; Decoteau *et al.*, 1988) increases as a result of a short irradiation with FR at the end of the photoperiod in a WL/D cycle. This effect is reversed by a subsequent WL pulse, indicating that P functions as the photoreceptor. A pronounced end-of-day FR response was observed in both wild type and the *au* mutant (Fig. 7.3). Therefore P must be present and functional in light grown plants of the *au* mutant. Since the deficiency in the *au* mutant lies in its ability to accumulate *l*P it is proposed that *s*P, quantitatively similar in *au* mutant and wild type, is responsible for the end-of-day FR effect.

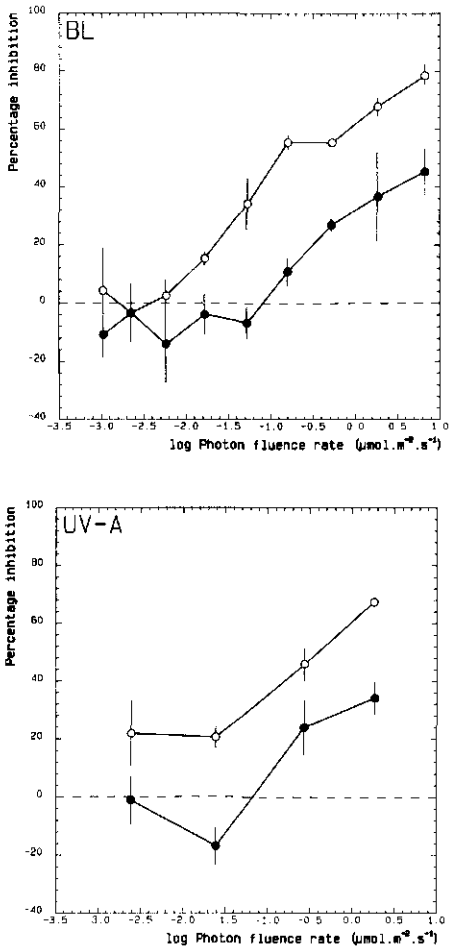

Fig. 7.2. Fluence-rate response curves for inhibition of hypocotyl growth of the *au* mutant (•) of tomato and its isogenic wild type (o) grown for 24 h in (**above**) blue light (BL) and (**below**) UV-A of different photon fluence rates at 25 °C. Seedlings were grown for 5 d (wild type) or 6 d (*au* mutant) in darkness before transfer to the light. Results are presented as % inhibition $(1 - [L_l - L_i / L_d - L_i]) *100$, where L_i = length prior to the light treatments, L_d = length after another 24 h D and L_l = length after 24 h irradiation. The actual growth of the dark controls over the 24 h period ($L_d - L_i$) for BL was 8.9 ± 0.7 mm for the wild type and 9.3 ± 0.7 mm for the *au* mutant and for UV-A 9.9 ± 0.5 mm for the wild type and 9.7 ± 0.9 mm for the *au* mutant.

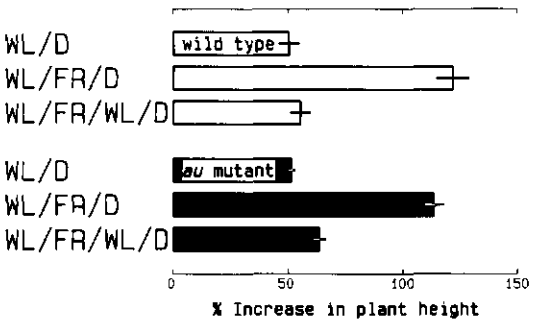

Fig. 7.3 End-of-day far-red light (FR) response of stem elongation in *au* mutant of tomato and its isogenic wild type at 23 °C. Plants grown for 28 d in daily white light (WL; 35 W m^{-2} [PAR, 160 μmol m^{-2} s^{-1}]; 14 h) / dark (D; 10 h) cycles were treated for 5 d with 20 min FR (20 μmol m^{-2} s^{-1}); 20 min FR immediately followed by 10 min WL; or D at the end of the daily light period. Results are presented as % increase in height of the plants during the end-of-day FR treatment. The actual increases in height were: wild type: D, 43 mm; FR, 76.3 mm; FR/WL, 45.2 mm; *au* mutant: D, 21.5 mm; FR, 46.5 mm; FR/WL, 22.7 mm.

7.3.4 Simulated phototropism

When one cotyledon is covered with aluminium foil and the plants are irradiated with WL from above, both wild type and *au* mutant curve towards the uncovered cotyledon (37 ± 5 and 46 ± 7 degrees respectively). This response of de-etiolated seedlings due to RL absorption, presumably by P in the cotyledon, described earlier by Shuttleworth and Black (1977) for cucumber seedlings, is exhibited by both the *au* mutant and wild type. In view of the aforementioned end-of-day FR effect it is likely that this response in light-grown seedlings is an additional response regulated by *s*P.

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8. PHOTOCONTROL OF ANTHOCYANIN SYNTHESIS

8.1 Introduction

The biosynthesis of anthocyanins in plant tissues either requires light or is enhanced by it (Chapter 3). Interaction between two photoreceptors in the photoregulation of anthocyanin production in sorghum seedlings was reported by Downs (1964). Two photoreactions were observed: the first showed typical properties of a high irradiance response (HIR): high irradiances and exposure times of several hours were required. It had a maximum sensitivity in the blue light (BL) spectral region at about 470 nm. The second photoreaction controlled the first one and showed a typical phytochrome (P) response, requiring short exposure times and exhibiting red light (RL)/far-red light (FR) reversibility. Recent studies have confirmed and extended these findings (e.g. Vince and Grill, 1966; Mancinelli, 1983; 1985; Mohr *et al.*, 1984; Mohr, 1980; 1986; Sponga *et al.*, 1986). The nature of the interaction between the two photoreactions is still unknown, but a possible mode of co-action has been suggested by Oelmüller and Mohr (1985). Their scheme describing this suggested mode of co-action has been presented in Chapter 3 (Fig. 3.1). Pfr is suggested as the terminal effector involved in the photoregulation of anthocyanin synthesis, whereas the light-dependent establishment of responsiveness towards Pfr is mediated by P itself, a BL/UV-photoreceptor (cryptochrome) or a UV-B photoreceptor. The interaction between cryptochrome and P in the photoregulation of anthocyanin varies between species. Several species have an absolute requirement for a pretreatment with BL or UV, whereas in other species FR pretreatments are equally effective as BL or UV (Chapter 3). In tomato it appears that this interaction is not obligatory, since pretreatment with RL or FR, absorbed by P and not by cryptochrome, is also effective. However, RL and FR are less effective than BL or UV. When continuous irradiation was applied to induce anthocyanin formation in tomato seedlings without an inductive pulse at the end of the light treatment, BL, UV-A and UV-B exerted a strong response, whereas FR and RL were only slightly effective (Drumm-Herrel and Mohr, 1982).

The experiments described in this chapter were designed to achieve a better understanding of this interaction in tomato by utilizing mutants with either an enhanced capacity for anthocyanin synthesis (the *high pigment*, *hp* mutant) or a deficiency in labile (*l*) P (the *aurea*, *au* mutant).

8.2 Materials and methods

8.2.1 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). The mutant has been identified as a photoreceptor mutant (Chapter 4): the amount of P in etiolated seedlings of the *au* mutant is < 5% of that detected in etiolated wild type.
The Moneymaker *hp* mutant used in these experiments was not completely isogenic with the wild type and was derived from a cross between Moneymaker wild type and Webb Special (= *hp* mutant, LA 279), described by several authors (Kerr, 1960; Thomson *et al.*, 1962) as a spontaneous mutant with an exceptionally high anthocyanin content. The *au/hp* double mutant has been isolated from crossings between these *au* and *hp* mutants. The overall morphology of the *au/hp* recombinant resembled the *au* phenotype (Fig. 8.1.). In addition seeds produced by selfing mutants, *au* and *hp*, and their isogenic wild type of the cv. Ailsa Craig (obtained from the Glasshouse Crops Research Institute, Littlehampton, U.K.) were used.

8.2.2 Anthocyanin assay

Seeds were surface sterilized for 5 min in a 1% (v/v) 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 darkness (D) at 25 °C the irradiation schedule was started. Each treatment was terminated with a 24 h D period, after which anthocyanin extraction and assay was started. Samples of 10 seedlings of uniform height were taken from each box and extracted with 1.2 ml acidified (1 % HCl, w/v) methanol for 48 h in D with shaking. A Folch partitioning 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. In most experiments the results represent the mean ± S.E. of 4 replicates for each treatment and usually are the means of two or more independent duplicate experiments.

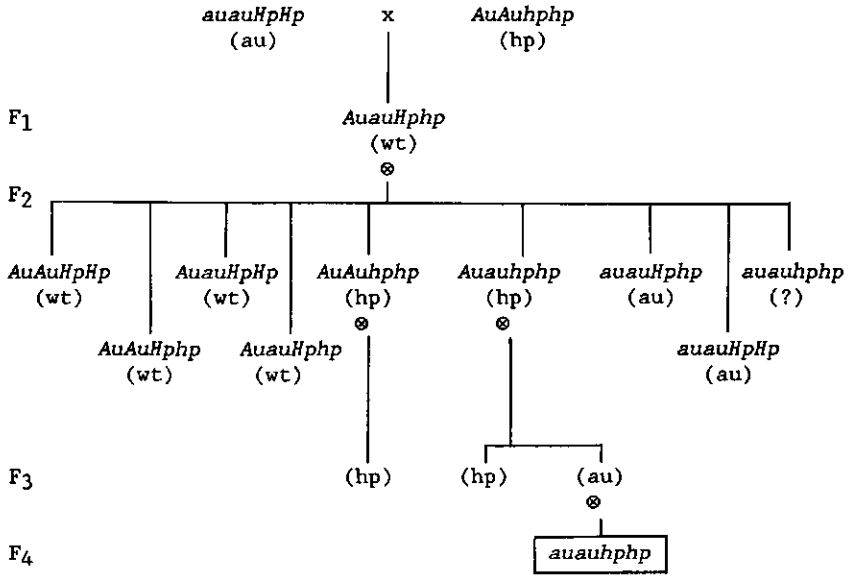

Fig.8.1. Scheme describing the isolation of the double mutant (*au/hp*) of tomato without an *a priori* prediction of its phenotype. Between brackets description of the phenotype and conditions used for selection (wt = wild-type seedlings with almost white hypocotyls after 24 h low irradiance red light (RL), short hypocotyls in white light (WL); au = no visible anthocyanin after 24 h low-irradiance RL, long hypocotyls in WL and reduced chlorophyll content; hp= etiolated seedlings with red hypocotyls after 24 h low-irradiance RL, short hypocotyls in WL. (x = crossed; ⊗ = selfed)

8.2.3 Light sources

The broad-band light sources for BL, RL and FR are described in appendix 'Light Sources'. The fluence rate of the BL, used as pretreatment, and of the RL and FR pulses was 3.6 μmol m^{-2} s^{-1} ± 5%.

8.3 Results and discussion

After a 12 h BL pretreatment, anthocyanin was produced in both the *hp* mutant and the wild type (Fig. 8.2a). A pulse of RL terminating the BL pretreatment

resulted in a stimulation of anthocyanin synthesis and a pulse of FR or RL followed by FR reduced anthocyanin synthesis below the dark-control level (BL pretreatment only). However, the response of the *hp* mutant was much larger. The *au* mutant exhibited no significant anthocyanin synthesis under these conditions (Fig. 8.2.a).

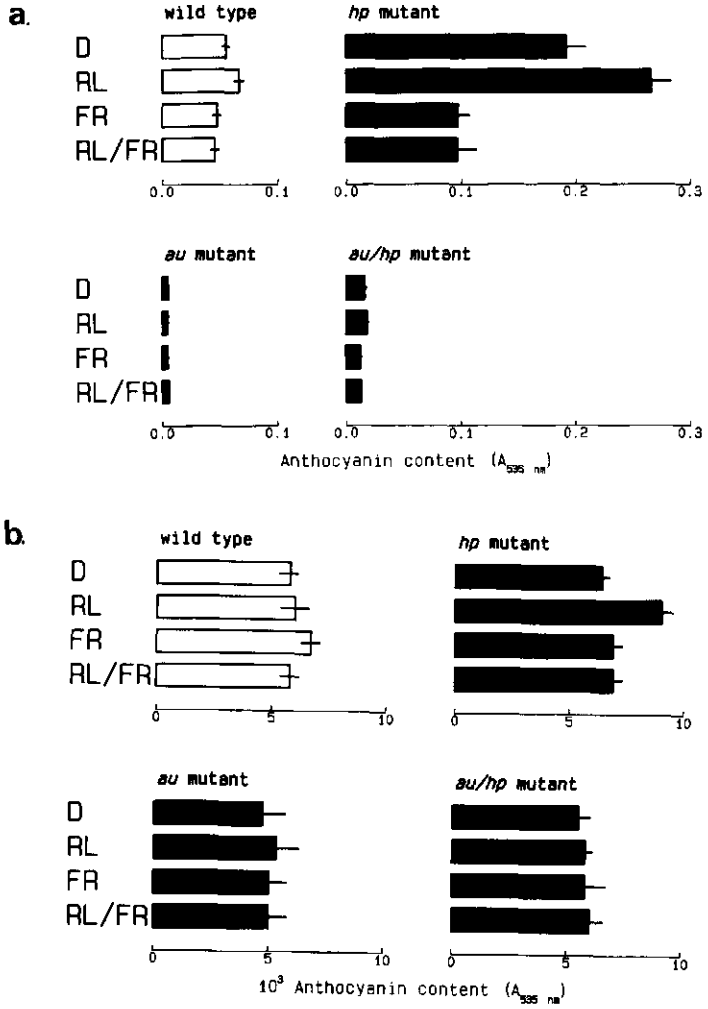

Fig. 8.2. Anthocyanin content of tomato seedlings of cv. Moneymaker *au*, *hp*, *au/hp* and wild type after treatment with a pulse of red light (RL; 5 min, 3.6 μmol m^{-2} s^{-1}), far-red light (FR; 15 min, 3.6 μmol m^{-2} s^{-1}) or RL followed by FR:
a. with 12 h blue light (BL) pretreatment (3.6 μmol m^{-2} s^{-1});
b. without BL pretreatment. Note the different absorbance scales.

Without BL pretreatment no significant P control of anthocyanin synthesis could be detected in seedlings of the cv. Moneymaker wild type and *au* mutant. However, the *hp* showed a small, but significant, induction of anthocyanin synthesis after a single RL pulse. This response to RL was reversed by a subsequent FR pulse (Fig. 8.2.b). Similar results were obtained with the wild type and *hp* mutant of the cv. Ailsa Craig (Fig. 8.3.a and b). These results indicate that the conclusion of Drumm-Herrel and Mohr (1982) 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. In

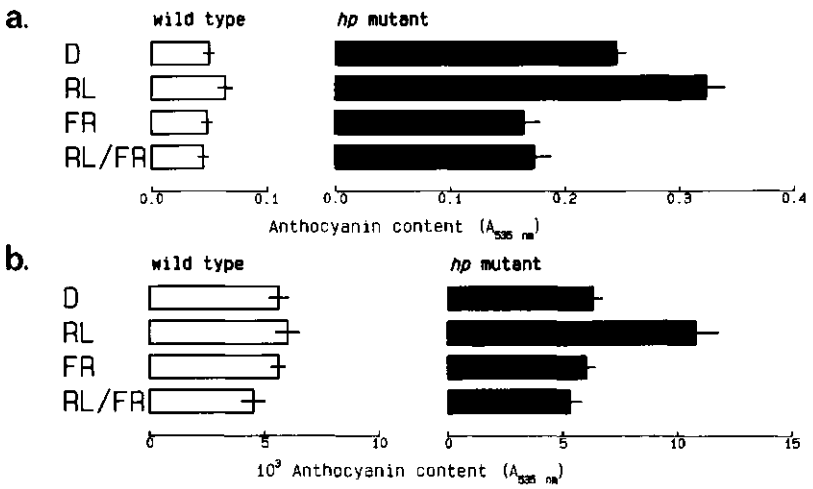

Fig. 8.3. Anthocyanin content of tomato seedlings of cv. Ailsa Craig *hp* and isogenic wild type after treatment with a pulse of red light (RL; 5 min, 3.6 μmol m^{-2} s^{-1}), far-red light (FR; 15 min, 3.6 μmol m^{-2} s^{-1}) or RL followed by FR:
a. with 12 h blue light (BL) pretreatment (3.6 μmol m^{-2} s^{-1});
b. without BL pretreatment. Note the different absorbance scales.

the *hp* mutant this effect was clearly detectable due to its enhanced capacity for anthocyanin synthesis. The cause of this enhanced synthesis is still unclear. Preliminary measurements of P in D-grown seedlings indicated no differences in P content between wild type and *hp* mutant (Table 4.2 in Chapter 4) and therefore the difference does not appear to be due to a higher [Pfr] in the *hp* mutant. On this basis the *hp* mutant was provisionally

designated as a photoresponse mutant (Chapter 2). However, another possible explanation of the increased anthocyanin synthesis is that the sensitivity of the *hp* mutant to Pfr is increased. A comparison of the photo-inhibition of hypocotyl elongation of wild type and *hp* mutant could provide useful information about possible differences in general sensitivity to Pfr. An indication of this has been observed by von Wettstein-Knowles (1968). The *hp* mutant used in their research had a lower hypocotyl dry weight than the wild type when grown in the light. Similar results have been shown by Mochizuki and Kamimura (1984). This points to increased inhibition by light and could indicate that the *hp* mutant has a higher sensitivity to Pfr than wild type. Perhaps more likely is that the increase in anthocyanin synthesis is due to a mutation which influences a step much further along the transduction chain between photoreceptor and response, possibly the absence of an inhibitor (or a 'substrate-competitor') of a crucial reaction in anthocyanin synthesis. Whatever the final explanation, the *hp* mutant is a useful tool to study anthocyanin synthesis because of its 'amplified' response.

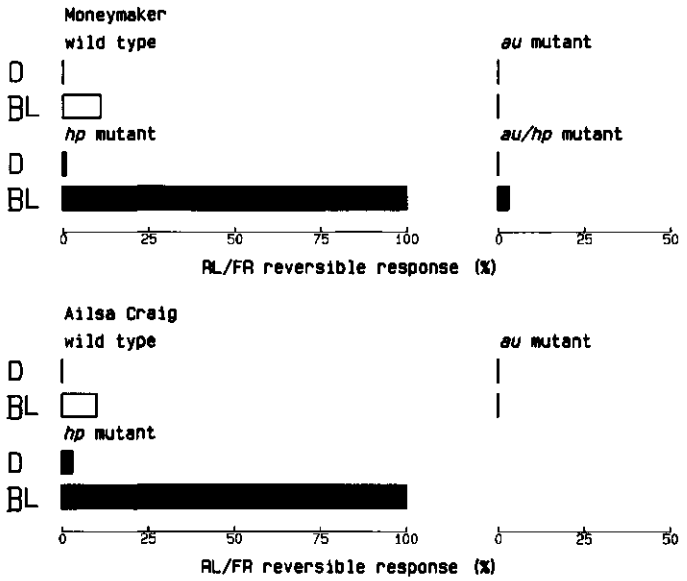

Fig. 8.4. The red (RL)/far-red light (FR) reversible anthocyanin synthesis after 24 h darkness (response to a RL pulse - response to a FR pulse) of tomato seedlings of *au*, *hp*, *au/hp* and wild type (cv. Moneymaker) and *au*, *hp* and isogenic wild type (cv. Ailsa Craig) expressed as percentage of the response in the *hp* mutant after a 12 h blue light (BL) pretreatment. All inductive treatments given at 84 h from sowing. RL: 5 min, 3.6 μmol m^{-2} s^{-1}; FR: 15 min, 3.6 μmol m^{-2} s^{-1}; BL: 12 h, 3.6 μmol m^{-2} s^{-1}.

Without pretreatment with BL no anthocyanin could be detected in the double mutant (*au/hp*). However, after a BL pretreatment this mutant showed a small (less than wild type), yet significant, RL/FR reversible response (Fig. 8.2.a). Apparently a low level of P below detection limits (either *l*P or stable P (*s*P)) that is present in the *au* mutant is now sufficient to induce a little anthocyanin due to the increased sensitivity as a result of the *hp* gene.

To enable comparison of the differences between the wild type and the three mutant genotypes a figure was constructed with the inductive RL response (response to RL pulse - response to FR pulse (= Δ R (Drumm-Herrel and Mohr, 1982))) expressed as percentage of the response of the *hp* mutant after BL pretreatment (Fig. 8.4.). It appears that the amount of anthocyanin in the *hp*, produced in 24 h D after a BL pretreatment, has been amplified about 10 fold as compared to the wild type. By extrapolation the inductive RL response after one RL pulse in the case of the wild type is about 0.1%. This would correspond to a difference in absorbance of $\approx 2.10^{-4}$, which is below detection limits. This explains why so far no effect of a single RL pulse on anthocyanin synthesis in tomato hypocotyls has been observed.

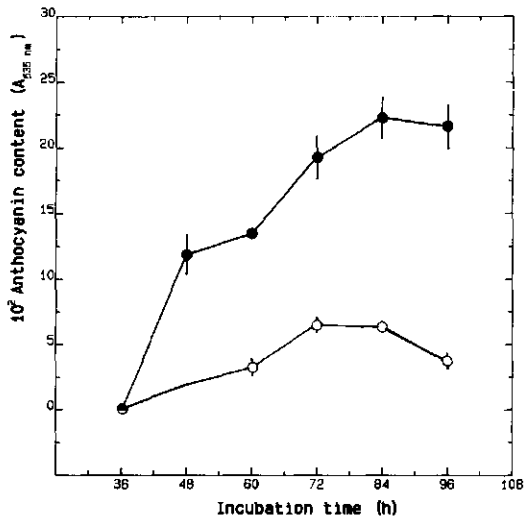

Fig. 8.5. Anthocyanin content of seedlings of tomato (cv. Moneymaker) wild type (o) and *hp* mutant (•) as a function of the dark (D)-incubation period preceding a 12 h irradiation with blue light (3.6 μmol m^{-2} s^{-1}) and terminated by 24 h D.

The anthocyanin synthesis in the *au/hp* mutant after a BL pretreatment is about 3% of the maximum response. The P level in the *au* mutant is < 5% of that in the wild type or the *hp* mutant (Chapter 4), i.e. the absence of at least 95% of the *1*P results in only 3% of the anthocyanin synthesis under P-control in the *hp* mutant after BL pretreatment. This clearly suggests that it is the 'bulk' *1*P pool in etiolated seedlings which is primarily involved in anthocyanin synthesis at this stage. Provisional observations indicate a little anthocyanin in older plants of the *au* mutant. It is possible that this is a response to the *s*P that accumulates in the light.

The 'timing' of the RL pulse appeared to be very important. In the case of the *hp* mutant the maximum inductive response to a single RL pulse with BL pretreatment was observed after 84 h D incubation (Fig. 8.5). Apparently at this time the seedlings have acquired maximum competence to respond to Pfr. Two pulses of RL, the first after 72 h D and the second

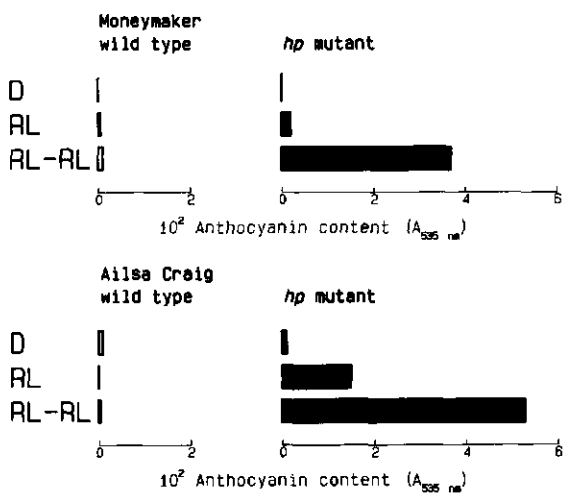

Fig. 8.6. Anthocyanin content of tomato (cv. Moneymaker and cv. Ailsa Craig) seedlings of wild type and *hp* mutant following one or two inductive light pulses. The first pulse of red light (RL; 5 min, 3.6 μmol m^{-2} s^{-1}) was given after 72 h incubation in darkness (D) and the second at 84 h. Between and after the pulses the seedlings were kept in D until the anthocyanin determination at 96 h after sowing.

after a further 12 h D, were much more effective than one RL pulse in

induction of anthocyanin synthesis during a 24 h D period in the case of the *hp* mutant (Fig. 8.6). Wild type under the same conditions showed no significant anthocyanin accumulation. Using the model proposed by OelmÀller and Mohr (1985) (Fig. 3.1, Chapter 3) this can be explained as follows: P 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 other words in determining the responsiveness of the anthocyanin synthesis towards Pfr. This responsiveness can also be established by a pretreatment with BL or UV. The first RL pulse is proposed to increase the sensitivity to Pfr, after which the inductive response of

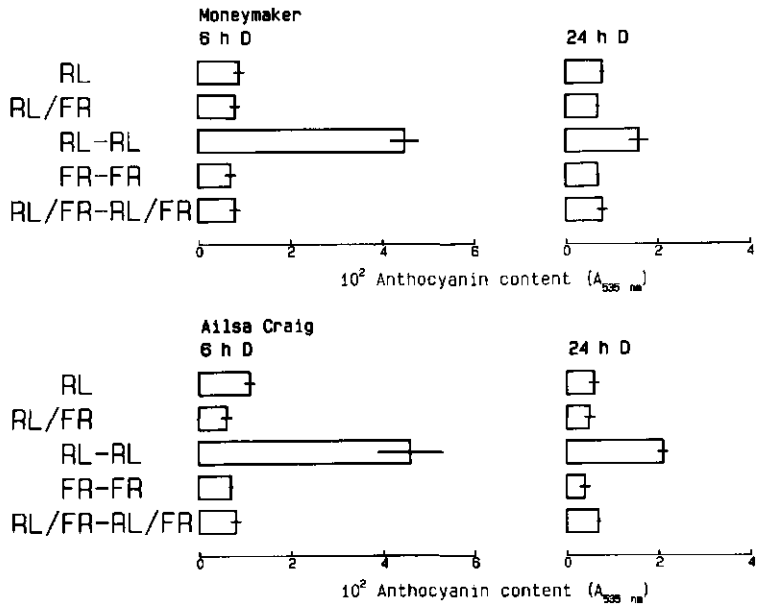

Fig. 8.7. Anthocyanin content of tomato (cv. Moneymaker and cv. Ailsa Craig) seedlings of *hp* mutant following one or two inductive light pulses. The first pulse was given after 60 h incubation in darkness (D) (with 24 h D between two pulses) or after 84 h incubation in D (with 6 h D between two pulses) and the second at 90 h: one or two pulses of red light (RL; 5 min, 3.6 μmol m^{-2} s^{-1}); one or two pulses of far-red light (FR; 15 min, 3.6 μmol m^{-2} s^{-1}); two pulses of RL followed immediately by FR (RL/FR). All treatments were terminated with a 24 h D period before anthocyanin determination.

the second pulse is enhanced. A treatment of two pulses of RL separated by a

D period of 24 h was less effective than pulses with a 6 h D interval (Fig. 8.7). Apparently the increased responsiveness to a second pulse is gradually lost if the D period is too long. This is in similar to the observation of Mohr and Drumm-Herrel (1983), who showed that the 'sensitivity amplification' to Pfr induced by UV-B for anthocyanin synthesis of wheat coleoptiles was completely lost if 12 h D separated the UV-B treatment and the inductive RL pulse.

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9. GENERAL DISCUSSION

Physiological mutants offer a wide range of new possibilities in assisting the study of higher plant photomorphogenesis. In the experiments described in this thesis three such mutants have been used. In this chapter general conclusions are presented, together with additional discussion, speculations and suggestions for future research.

The mutants concerned are the long hypocotyl (*lh*) mutant of cucumber and the *aurea* (*au*) and high pigment (*hp*) mutants of tomato. Spectrophotometric as well as immunological evidence indicate that the *au* mutant lacks labile phytochrome (*l*P), whereas the *lh* mutant has been proposed to lack stable phytochrome (*s*P). While the *au* mutant appears to be a *l*P receptor mutant, the *lh* mutant could be a *s*P receptor mutant. However, confirmation awaits the availability of species-specific antibodies which can discriminate between *l*P and *s*P. In Figure 9.1 the possible complementary nature of the mutants is visualized. Since the level of *s*P in etiolated

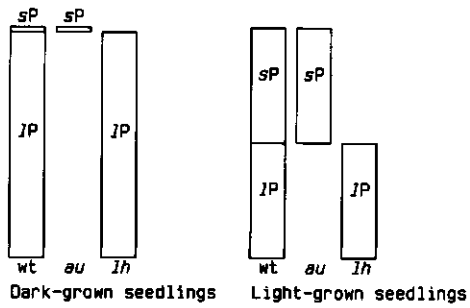

Fig. 9.1. Schematic illustration of the proposed phytochrome (P) content (labile: *l*P and stable: *s*P) of dark-grown and light-grown seedlings of wild type, tomato *au* mutant and cucumber *lh* mutant. Note that the total P level in light-grown seedlings is approximately 2% of the level in dark-grown seedlings.

tissue is too low to measure spectrophotometrically, the phytochrome (P) level in dark-grown seedlings of the *lh* mutant appears similar to that in the wild type, whereas in the *au* mutant no P can be detected. Despite the low level of P in light-grown tissue, both mutants were demonstrated by spectrophotometry to contain about 50% of the P present in the wild type. The P detected is proposed to represent *s*P in the the *au* mutant and *l*P in

the *lh* mutant.

One of the first problems that can be addressed with these mutants is: which of the two forms of P is physiological active? Formation of the far-red light (FR)-absorbing form of P (Pfr) has been proposed to be the physiologically active process in P action. Alternatively it has been postulated that it is the loss of the red light (RL)-absorbing form (Pr) which is the active process (Smith, 1983). Circumstantial evidence has implicated the Pfr form of P as the physiologically active form (Borthwick *et al.*, 1952), but mutants lacking P provide, for the first time, direct evidence to support this hypothesis. Mutants such as *au* mutant of tomato clearly have a reduced level of Pr in dark-grown seedlings, but are elongated in darkness (D), like the wild type. If removal of Pr was the active process in P action the mutant would be expected to be short. Mutants possessing different levels of P, would also be very useful in determining whether a response is due to the [Pfr] or Pfr/Ptotal ratio (φ). Until now different P levels were only attainable by various pre-irradiation schedules, which may selectively influence subsequent response sensitivity.

Why do plants posses a bulk *I*P pool in dark-grown plants? Hillman (1972) attempted to attribute function to this pool, but concluded: "As to what the function might be, there is simply no evidence". Paradoxes which remain unexplained are the *Zea* and *Pisum* paradoxes. In *Zea*, a fluence of RL sufficient to saturate a particular physiological response is insufficient to cause a detectable conversion of P to Pfr. This response is apparently saturated by a very small amount of Pfr. However, the effect of the RL is reversible by a saturating FR pulse, even though the FR establishes a measurable conversion of Pr to Pfr, in fact more than the RL which effect it reverses. This apparent paradox has been explained in terms of a limited number of localized receptor sites to which Pfr migrates (Raven and Spruit, 1973; Hendricks and VanDerWoude, 1983; Kraak, 1986). The P molecules bound at these sites therefore consist of 100 % Pfr. This is higher than is possible on photochemical grounds alone. It was further proposed that low fluence FR, as well as RL, produced sufficient Pfr to saturate the receptors and initiate a response. Assuming that the bound P molecules reside at the receptor sites, even if photoconverted back to Pr, the explanation of the VLFR by longer irradiation periods is possible. FR causes the photoconversion of most Pfr at the receptors to Pr, resulting in a low photoequilibrium of the P at the receptors ($\varphi < 0.05$) and reversion of the response.

In *Pisum* a paradox is observed when tissues from plants, pre-irradiated with RL and then kept in D, are later briefly irradiated with sources establishing various photostationary states (φ) and then returned to D. Those given a low φ value grow more than the corresponding dark controls (pre-irradiated only), while those given high φ values grow less. A response similar to the dark controls is obtained by brief irradiation establishing a φ value of about 0.20. Thus, on this physiological basis, the pre-irradiated tissue responds as though 20% of its P was Pfr. However, no Pfr could be detected spectrophotometrically. Here again, as in *Zea*, the existence of a small active fraction of P with properties different from the bulk pool could explain this paradox. In this case there has to be a difference between the pools in the rate(s) of Pfr reversion and/or destruction after RL irradiation. The active fraction could either revert more slowly or undergo a slower rate of destruction than the bulk P.

It is possible to speculate that the small active pool is *s*P. However, this implies that the bulk P pool, *l*P, is non-functional. It seems 'highly inefficient' for the plant to expend energy, while living heterotrophically on a limited food reserve, to make a large pool of a photoreceptor which is non-functional. Kendrick and Spruit (1973) concluded: ".... all P can be proposed to be potentially active and to trigger specific responses by virtue of its localization in a particular environment".

Experiments with the *au* mutant, lacking *l*P, have now provided direct proof that the bulk pool of *l*P is indeed functional in dark-grown plants. At the fluence rates tested the *au* mutant is more or less 'RL blind'. Fluence rate response curves for BL demonstrate that the *au* mutant requires higher fluence rates to obtain the same inhibition of growth as the wild type. Since this mutant contains at most 5% of the P present in wild type it was concluded that it was the absence of 95% *l*P that causes this difference. This provides evidence that the bulk pool of *l*P is active in the control of hypocotyl elongation. It has to be kept in mind that it still remains possible that *s*P is also reduced in the *au* mutant in the same proportions as *l*P, causing the difference in fluence rate requirement described above. However, the evolutionary argument that *l*P is functional at a time that energy is at premium seems overwhelming. Precise determinations of the exact *s*P concentrations of dark- and light-grown tissues are clearly needed. However, this is impossible using spectrophotometric methods. The development of new antibodies, which are capable of discriminating between *s*P and *l*P, is required. These would enable accurate determination of *s*P and

*1*P levels.

Considering the selective pressures for the evolution of light perception in dark- and light-grown plants the existence of a large pool of *1*P can be explained. The dark/light transition (de-etiolation) of a seedling takes place as the seedling approaches the soil surface. Until light is perceived the strategy of the seedling is to grow as rapidly as possible to reach the surface using the stored reserves as energy source. This has been called the strategy of dark-growth or scotomorphogenesis (Mohr and Schäfer, 1983). When the seedling perceives light a new strategy is adopted: photomorphogenesis. The process of de-etiolation takes place and the seedling starts using light as an energy source. To enable switching as quickly as possible from scotomorphogenesis to photomorphogenesis after emerging from the soil (or more precisely after anticipating the soil surface a few mm below it) higher plants have evolved a very sensitive photoperception system. High sensitivity could be accomplished in several ways:

(i) The presence of a high concentration of P in the seedling. This would increase the chance that the first few photons penetrating the soil would convert some Pr molecules to Pfr which could then move to a limited number of receptors and lead to de-etiolation.

(ii) A more sensitive P system. In this case a few Pfr molecules would suffice to induce a response. This could be accomplished by the presence of a small number of receptors for Pfr. This type of response is consistent with the very low fluence response (VLFR). In such responses the photoequilibrium established by a pulse of FR results in sufficient Pfr molecules to saturate the response. Such responses lack RL/FR reversibility: the character by which P involvement in a response is implicated. In these cases only the action peak in the RL region of the spectrum implicates Pr as photoreceptor in the VLFR (e.g. Blaauw-Jansen and Blaauw, 1976; Cone and Kendrick, 1985).

After emerging from the soil this high sensitivity is no longer necessary and the primary function of P is the detection of canopy shade. The seedling now requires a system able to accurately monitor changes in light quality, e.g. the RL/FR ratio (Smith, 1986). A small pool of a RL/FR-reversible photoreceptor could fulfil this function. It is interesting to note that from an evolutionary standpoint, P probably evolved for this function and that its utilization to detect the dark/light transition is a more recent adaptation. In other words, *1*P is a later evolutionary

development associated with land plants. What the relationship between *l*P to *s*P is remains an open question, but at this time it is uncertain as to wether *l*P is a separate gene product or a processed form of *s*P.

The availability of the *au* mutant of tomato, with less than 5% of the P present in wild type, enables the the involvement of P in a VLFR to be further studied. The amount of P in this mutant could be so low that a FR pulse fails to convert enough Pr molecules to Pfr to induce a VLFR and thus bring the VLFR within the range of RL/FR reversibility. Indeed such reversibility of a VLFR could explain the observations of Sharrock *et al.* (1988) in the *au* mutant. The induction of the Chl *a/b* binding protein (*cab*) has been demonstrated, at least in part, to be under the control of a VLFR in a number of species (Kaufman *et al.*, 1984). In the *au* mutant it appears that the low level of induction of *cab* by RL is reversible by FR, while in the wild type *cab* is strongly induced by FR. This result provides the first indication that *l*P is the functional photoreceptor in VLFR's.

It is interesting to note that the adult plants of the *au* mutant have a reduced chlorophyll content compared to the wild type and that this remains the most striking feature of the *au* phenotype (Koornneef *et al.*, 1985). One can speculate that the low level of *l*P present in light-grown plants of a tomato wild type retains the function of regulating chlorophyll biosynthesis, just as it does at the dark/light transition of a seedling at the time of de-etiolation (Koornneef *et al.*, 1985; Sharrock *et al.*, 1988).

Whereas P control of hypocotyl elongation in etiolated seedlings of the *au* mutant is reduced by lack of *l*P, elongation growth of light-grown seedlings appears still to be under P control. The classic end-of-day FR response is clearly present in the *au* mutant, indicating that P is present and functional. The *s*P type of P is probably involved in this response. Additional evidence in support of this hypothesis comes from studies with the *lh* cucumber mutant. This mutant, which is proposed to lack *s*P or its function, shows no end-of-day FR response. Furthermore, whereas elongation of the internodes of the tomato *au* mutant is similar to that in its wild type when grown under greenhouse conditions, pointing to (*s*)P control, the cucumber *lh* mutant has internodes much longer than its wild type, indicating absence of P control in light-grown plants.

Apart from offering the possibility of ascribing functions to the different P types, mutants also enable the study of the relative importance of photoreceptors, for instance in the coaction between P and BL/UV-photoreceptor. Fluence-rate response curves for inhibition of hypocotyl

growth by BL clearly show that inhibition is, at least in part, mediated by *1*P. However, a significant inhibition by BL is still observed in the *au* mutant. Mohr and Drumm-Herrel (1983) have proposed that the function of the BL/UV-photoreceptor is to enhance sensitivity to P. On this basis, the higher BL fluence rate required in case of the *au* mutant to obtain the same inhibition as wild type could be required to increase the sensitivity sufficiently to compensate for the reduced P content (Fig. 9.2). Irradiation of the wild type with 0.38 μmol m^{-2} s^{-1} BL causes 50% inhibition. Assuming the *au* mutant contains 5% of the P present in wild type, the same inhibition

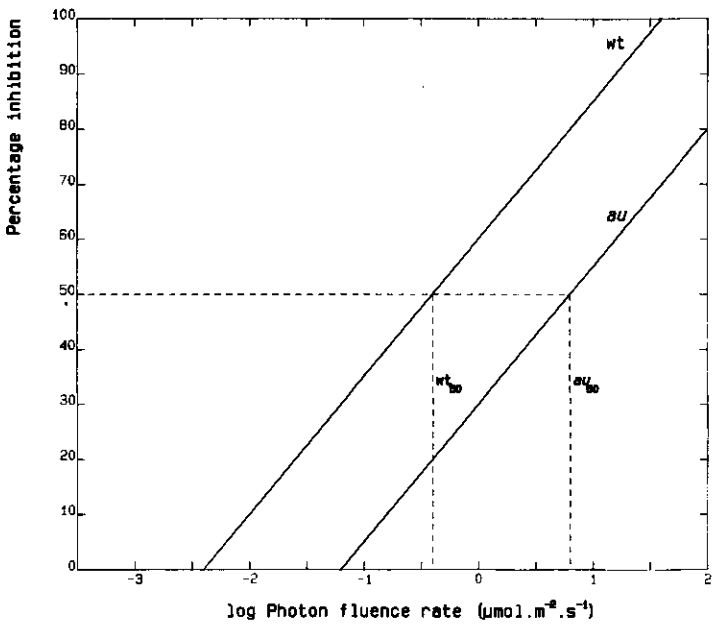

Fig. 9.2. Schematic illustration of the fluence rate dependency of inhibition of hypocotyl elongation by blue light (BL) in etiolated seedlings of tomato *au* mutant and wild type (wt). The fluence rates at which inhibition of *au* mutant and wild type is 50% are indicated by au_{50} and wt_{50}, respectively. Based on the data of Fig. 7.2.

would occur in the mutant when irradiated with 7.8 μmol m^{-2} s^{-1} BL (= 20 x 0.38). In fact 50% inhibition of the *au* mutant occurs after irradiation with 13.2 μmol m^{-2} s^{-1} BL (= 35 x 0.38). This calculation would indicate a P content in the *au* mutant $\approx$ 3% of that in wild type.

Theoretically it could be predicted that seedlings of the *au* mutant at higher BL fluence rates would respond to P control to the same extent as the wild type. To verify this hypothesis a study of P responses after a BL pre-irradiation or by manipulation of φ by supplementary irradiation during a BL irradiation is necessary. Comparable experiments were conducted by Drumm-Herrel and Mohr (1984) with seedlings of *Sesamum indicum*, indicating that the growth rate after the end of a BL period can be fully attributed to the action of Pfr, as long as the responsiveness amplification by BL persists in the subsequent D period. They observed that the responsiveness decreases during the D period. To detect P-controlled growth responses before sensitivity is lost very sensitive measuring techniques are required. The apparatus for continuous measuring of growth, described in Chapter 6, will enable such experiments to be conducted.

One observation does not appear to be consistent with this hypothesis. Continuous irradiation with white light (WL) should enhance the sensitivity to P due to the BL component present and thus enhance the P response. However, the hypocotyl of the *au* mutant grows long in WL. Drumm-Herrel and Mohr (1984) have argued that the BL component in the WL used in such experiments is possibly below the threshold necessary to obtain a detectable response. When WL is given at a fluence rate of 32 μmol m^{-2} s^{-1}, this contains approximately 3 μmol m^{-2} s^{-1} BL (as described in Chapter 5, Fig. 5.7). This fluence rate would only cause a small growth inhibition in the *au* mutant as can been seen in Figure 9.2 (see also Chapter 7, Fig. 7.2). Furthermore, the higher φ value in WL ($\approx$ 0.72) as compared to the φ value in BL ($\approx$ 0.45) would increase the response, i.e. the threshold in WL should be lower than the threshold in BL. It is thus unlikely that the fluence rate of the BL component of the WL in these experiments was below the threshold.

Coaction between P and a BL/UV-photoreceptor also plays a role in anthocyanin synthesis. As with hypocotyl elongation it has been proposed that the function of both P and the BL/UV-photoreceptor in this response is to enhance the sensitivity of the system to Pfr (Oelmüller and Mohr, 1985). Without enhanced sensitivity induced by a pre-irradiation no P controlled anthocyanin synthesis could be detected in tomato wild type seedlings after an inductive light treatment. However, in the tomato *hp* mutant P control of anthocyanin synthesis could be detected without a pre-irradiation. This *hp* mutant appears to have an enhanced capacity for anthocyanin synthesis. It was proposed, that this mutant has an increased sensitivity to Pfr. Experiments are in progress to test this hypothesis. In the *au/hp* double

mutant anthocyanin synthesis could only be detected after a BL pretreatment. The sensitivity amplification induced by the *hp* mutation was not sufficient to induce a measurable amount of anthocyanin synthesis in seedlings containing a very low P level. Furthermore, anthocyanin synthesis in the *hp* mutant after a BL pretreatment followed by a RL pulse was four times higher than without a BL pretreatment. This indicates that the effects of the BL pretreatment and the *hp* mutation were, at least partially, additive.

It appears that anthocyanin synthesis in etiolated tomato seedlings, similar to P control of hypocotyl elongation, is also controlled by *1*P. The most likely explanation of the small response in the *au/hp* double mutant is a response to a low level of *1*P present. The existence of a low level of *1*P in the *au* mutant is probably the reason why it can survive. A mutant lacking all *1*P would probably be lethal since it could not undergo the process of de-etiolation. This hypothesis can be enforced with the following observations. The *hp* mutant contains a P level similar to the wild type, whereas the *au* mutant and the *au/hp* double mutant contain at most 5% of the P level in wild type. Anthocyanin synthesis, induced by pre-irradiation with BL and an inductive RL pulse, increases 10 fold due to the *hp* mutation. If the RL/FR-reversible response of the the *hp* mutant after a BL pretreatment is taken as 100%, then it is predicted that the responses of wild type, *au/hp* double mutant and *au* mutant will be 10%, 5% and 0.5%, respectively. These values were indeed measured in the experiments described in Chapter 8. The RL/FR-reversible response of the *au/hp* double mutant was even less: 3%, which could indicate that the P level was ≈ 3% of that in wild type. Such a prediction is compatible with the results of hypocotyl inhibition in continuous BL.

The *hp* mutant with its enhanced anthocyanin synthesis offers an excellent possibility to study P control of anthocyanin in tomato seedlings. This will be done in a forthcoming programme of the research group. The distribution of anthocyanin in the seedlings then will be studied in detail. Preliminary results indicate that this differs with different light treatments. It is possible that the study of the location of several anthocyanin 'areas' (e.g. in the hypocotyl base or in the hypocotyl hook) in a seedling, depending upon age or light treatment, will reveal information about the capability of the different tissues to synthesize anthocyanin. At present it remains a mystery as to how or why one cell is capable of anthocyanin synthesis whereas another is not.

The function of P during de-etiolation includes the regulation of gene

expression. A number of enzymes have been shown to appear or be dramatically increased as a result of *de-novo* synthesis following photoconversion of P. The *au* mutant has been utilized to attribute function, for the first time, to *IP* in the control of gene expression. Modulation of the Chl *a/b* binding protein (*cab*) is severely reduced in the *au* mutant (Sharrock *et al.*, 1988). Using the mutants described in this thesis it should be possible to study the P control of expression of other genes.

Using genetic engineering techniques new possibilities of photomorphogenetic mutants arise: transgenic plants. With the use of a gene transfer (transformation) system a foreign gene can be inserted into the plant genome and be functional in its new location. Effective transformation procedures have been developed that use *Agrobacterium tumefaciens* as a vector (Horsch *et al.*, 1985) or that introduce DNA directly into protoplasts (Paszkowski *et al.*, 1984). There are several ways in which transgenic plants have already added and will continue to add to the understanding of photomorphogenesis in higher plants:

(i) The study of light regulation at the gene level. A number of light regulated genes have been studied in detail (Jenkins *et al.*, 1983; Quail *et al.*, 1987; Tobin, 1987; Aoyagi *et al.*, in press; Kuhlemeier *et al.*, in press; and references therein) including the P gene (Vierstra and Quail, 1986). A number of these genes have up-stream sequences which are important in controlling photoregulation of the transcription process. Mutants in which such sequences have been modified are providing useful tools in identifying the functional DNA sequences (Hanley-Bowdoin and Chua, 1987). These up-stream sequences have been isolated and introduced into reporter genes which are not normally light-regulated (Kuhlemeier *et al.*, 1987; Strittmatter and Chua, 1987; Nagy *et al.*, 1988). In this way photo-inducibility has been conferred upon these genes.

(ii) The construction of mutants with anti-sense RNA. Once a given plant gene has been cloned it is possible to introduce a specifically modified form of that gene into a plant, where it is transcribed into a RNA strand complementary to the RNA produced by the normal functional gene (Weintraub *et al.*, 1985; Lichtenstein, 1988; Krol *et al.*, 1988). Binding of this anti-sense RNA to the natural RNA renders the latter non-functional. In this way it should be possible to 'titrate out' the mRNA from the P gene(s) and thus produce transgenic plants lacking P(s). Such techniques should provide a direct method for producing photoreceptor deficient plants without involving mutagenesis and mutant selection. Such genotypes would be

pleiotropic for the P action. If there are indeed two P types encoded by different genes without much sequence homology, this may be a way of producing plants deficient in one P type. This would permit the purification of the other type without contamination.

(iii) The design of specific selection schemes for P response mutants. Assuming that it is not P itself, but a so called 'trans-acting factor' that is part of the transduction chain of P, which interacts directly with light inducible promotors, it might be possible to select directly for mutations in these trans-acting factors. Schäfer (1987) and Karlin-Neumann and Tobin (1987) suggested constructing fusions of light-inducible promotors with a suicide gene and then after insertion of the fusion into the plant genome and a mutagenic treatment, selecting for mutants that survive. A possible candidate for such a suicide gene is the *tms2* gene from *A. tumefaciens* that, when active, makes a plant sensitive to α-naphaleneacetamide; a plant (mutant) with a non-functional *tms2* gene will be resistant (Karlin-Neumann and Tobin, 1987; Klee *et al.*, 1987). In addition one would expect in resistent plants alterations in (some) photomorphogenic responses.

Nagy *et al.* (1988) have suggested several other uses of plants transformed with specific constructs, that will help to understand the relevant molecular structure of P itself.

This thesis has provided proof of the usefulness of mutants in the study of photomorphogenesis at the whole plant level. Such mutants will also be of great benefit for scientists studying at the molecular level. Although recently emphasis has been on the use of molecular techniques, the study of whole plant physiology will remain indispensable.

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SUMMARY

Plants not only depend upon light as an energy source (photosynthesis), but also utilize it as a source of information, enabling growth and development to be tuned to the prevailing light environment (photomorphogenesis). A number of photoreceptor pigments have evolved enabling information to be sensed over the complete daylight spectrum. These include phytochrome (P), operating predominantly in the red (RL)/far-red (FR) spectral range, blue (BL)/UV-absorbing photoreceptor(s), operating in the BL spectral range and UV-B photoreceptor(s). These pigments detect parameters of the light environment, such as light quality, quantity, direction and duration, and enable developmental responses to be controlled accordingly. Many processes in plants are controlled by these photoreceptor systems, such as, germination, elongation growth, apical hook opening, enzyme activity, leaf expansion, phototropism and flowering.

The most extensively studied and characterized photomorphogenetic photoreceptor in higher plants is phytochrome. Recently the existence of multiple types of P has been suggested: labile P (*l*P) and stable P (*s*P). Where in darkness *l*P is predominant, whereas both *l*P and *s*P are present in similar quantities in the light. In addition to different P types there are also multiple working mechanisms of P: very low fluence response (VLFR), low fluence response (LFR) and high irradiance response (HIR). The BL/UV-photoreceptor(s) is/are hypothetical, being postulated on the basis of action spectra. The chemical composition is still unknown. The nature of the UV-B absorbing pigment(s) has also yet to be elucidated. The photoreceptors co-act in several processes. However, the mode of this co-action and the relative importance of the different photoreceptors is still unclear.

The existence of different photoreceptors, multiple photoreceptor types and working mechanisms means that photomorphogenesis is rather complex, especially because their absorption spectra often overlap. In this thesis it has been shown how mutants, lacking a particular component of their photomorphogenesis, can be used. The relevance of the deletion in the mutant is indicated by the difference between the mutant and its isogenic wild type. These mutants can be divided simplistically into three groups: photoreceptor-, transduction chain-, and response mutants (Chapter 1). Only a few photomorphogenetic mutants are known at the present, some of these are described in chapter 2. The mutants used in the present experiments were: the long-hypocotyl cucumber mutant (*lh*), and the *aurea* (*au*) and high pigment

(*hp*) tomato mutants. Both the *lh* mutant and the *au* mutant are characterized by an extremely elongated ('etiolated') hypocotyl when grown in white light (WL). The *hp* mutant shows an enhanced pigment synthesis, especially anthocyanin.

Spectrophotometric measurements indicated that the P content of etiolated seedlings of the *au* mutant was < 5% (= detection limit) of that in wild type (Chapter 4). Using immunochemical techniques, carried out in collaboration with Prof. P.H. Quail and coworkers (Plant Gene Expression Center, USDA, Albany, USA), this observation has been confirmed. It appeared that the P molecule was synthesized but not accumulated. Apparently it is unstable, but the reason why is not yet clear. In light-grown tissue of the *au* mutant with very low levels of chlorophyll (flower petals) or tissue bleached as a result of treatment with a herbicide (Norflurazon-treated leaves) the amount of spectrophotometrically detectable P was 50% of that in similar tissue of the wild type. It was proposed that the *au* mutant is a receptor mutant and that it lacks *l*P. However, detailed immunochemical determination using specific antibodies against *l*P and *s*P are required to confirm this conclusion. These antibodies are not yet available.

The P content of seeds and etiolated seedlings of the *lh* mutant appeared to be similar to that of the wild type. In light-grown tissue (flower petals and bleached leaves) of the *lh* mutant the P content was about 50% of that in the wild type. It is proposed that this mutant lacks *s*P or its function. Again, immunological determination is necessary to enable conformation of this conclusion. This research is in progress in collaboration with Prof. M. Furuya (Plant Biological Regulation, Frontier Research Programs (RIKEN), Saitama, Japan).

Using these mutants several photomorphogenetic processes have been studied. In chapter 3 a review is given of photomorphogenetic characteristics of tomato and cucumber as described in the literature. Furthermore, current theories are discussed in relationship to the photomorphogenesis of both species. The remaining chapters describe experiments comparing the characteristics of the mutants and their wild types.

In chapter 5 the effect of irradiation on long-term growth of cucumber seedlings is described. It appeared that the responses to different irradiations of etiolated seedlings of the *lh* mutant and its wild type are similar. After de-etiolation differences appear: the P control of elongation is reduced in the mutant and de-etiolation is retarded. In continuous WL or

RL the hypocotyl of the *lh* mutant is much more elongated than that of the wild type. The (epidermal) cells are also much longer, being increased in the same proportion as the hypocotyls. These results suggests that the difference between wild type and mutant lies in the presence or absence of *s*P.

Many species respond by increased elongation growth to a short irradiation with FR at the end of the photoperiod. Cucumber also shows this response. However, the *lh* mutants lacks this so called end-of-day FR response. This suggests that this response in light-grown seedlings is mediated by *s*P. An additional response possibly regulated by *s*P is 'simulated' phototropism. When one cotyledon is covered with aluminium foil and the plant is irradiated from above curvature towards the uncovered cotyledon occurs. This response of de-etiolated seedlings due to RL absorption, presumably by P in the cotyledons, is absent in the *lh* mutant.

Hypocotyl elongation of both wild type and *lh* mutant is inhibited by BL. However, the mutant is elongated in WL which contains a considerable BL component. Experiments suggest that the low BL fluence rates used could not sustain sufficient photosynthesis for growth. Furthermore, inhibition of growth by BL is possibly only temporary. In WL photosynthesis is sufficient to sustain growth and the *lh* mutant elongates. However, under these conditions the wild type is inhibited *via* P.

The long hypocotyl of the *lh* mutant is very similar to that of a wild type plant treated with gibberellin (GA). However, the proportional response for a given dose of GA_{4+7} is the same in wild type and *lh* mutant. Furthermore, whereas the *lh* mutant, compared to the wild type, has long hypocotyls in WL and RL, hypocotyl lengths of the *lh* mutant and the wild type are similar in BL or UV-A. However, the GA-mediated decrease of inhibition is similar in RL- and BL-treated seedlings, indicating that the influence of GA is independent from the effect of the light treatments. It therefore appears likely that the *lh* mutant is not a GA over-producer. However, at the present GA concentrations in *lh* mutant and wild type are unknown.

In the literature a clear difference between the kinetics of hypocotyl inhibition by BL and RL has been described. In order to detect such differences very sensitive measuring equipment is required, since growth rate changes can occur within minutes. Preliminary studies carried out with a horizontal microscope revealed that inhibition by BL occurred much more rapidly than inhibition by RL (Chapter 6). To enable very accurate growth

measurements under controlled environmental conditions an computer-controlled apparatus for continuous growth measurement was designed and constructed in the Laboratory for Plant Physiological Research. A detailed description of this apparatus is given in Chapter 6. Data-acquisition and analysis with a computer enables the comparison of many individual plants. Experiments with the growth measuring apparatus showed that inhibition of elongation of etiolated seedlings of both *lh* mutant and wild type by BL was observed after a few minutes, whereas the inhibition by RL was only observed after several hours. No significant differences occurred between etiolated seedlings of wild type and *lh* mutant. However, the results of long-term growth experiments suggest that there are clear differences between light-grown seedlings. At the present time no de-etiolated seedlings have been measured with the growth measuring apparatus.

The *au* tomato mutant, like the *lh* cucumber mutant, has an elongated hypocotyl when grown in WL. Furthermore, germination is delayed, the chlorophyll content reduced and only 5% of the P level present in wild type can be detected in the *au* mutant. It is proposed that this mutant contains no (or very little) *l*P (Chapter 4). In chapter 7 the results are described of experiments designed to study the role of *l*P in photomorphogenesis. The response of wild type and *au* mutant are compared for several photomorphogenetic processes.

Etiolated seedlings of the *au* mutant are more or less 'RL-blind'. This indicates a lack of P control in the mutant, apparently caused by the absence of *l*P. However, inhibition of hypocotyl elongation is still present in the *au* mutant, although a higher fluence rate is required. It appears that the 95% decrease of P content in the *au* mutant causes this difference. It is proposed that this *l*P, absent in the *au* mutant, plays an important role in hypocotyl elongation of etiolated seedlings. This provides direct evidence that *l*P is functional. Previously it was impossible to ascribe any function to *l*P, despite the fact that *l*P is present in 'bulk' amounts in etiolated seedlings. Two further experiments with the *au* mutant confirm conclusions drawn from experiments with the *lh* cucumber mutant. Both the end-of-day FR response, as well as the simulated phototropism were observed in the *au* mutant, suggesting that both processes are regulated by *s*P.

Anthocyanin synthesis is one of the processes controlled by P. Previous work suggested that one RL pulse is not sufficient to induce anthocyanin synthesis in tomato seedlings. Pre-treatment with BL or UV-A increases the sensitivity of the system towards P. However, experiments with the *hp* tomato

mutant, with its enhanced anthocyanin synthesis, indicate P control of anthocyanin synthesis after one RL pulse (Chapter 8). In the *au* mutant no anthocyanin could be detected, even after a pretreatment with BL. In the *au/hp* double mutant a significant amount of anthocyanin could be detected (about 3% of the amount synthesized in the *hp* mutant). This indicates that the 95% P absent due to the *au* mutation plays an important role in anthocyanin synthesis.

In the last chapter several general conclusions and points of discussion are presented. One of these conclusions is that Pfr is the active form of P. Until now no direct evidence was available concerning the physiological active process in P action: the increase of Pfr or the decrease of Pr. However, mutants with a reduced P content (and consequently less Pr) elongate in darkness to an extent similar to the wild type. If decrease of Pr was the active process they would be expected to be short. An additional conclusion is that *I*P, present in etiolated seedlings in 'bulk' amounts, is indeed functional. From an evolutionary standpoint this is understandable, since it would be very 'uneconomic' if a plant synthesized a large amount of a non-functional protein at a time when it is dependent upon limited food reserves.

The mutants described in this thesis appear to be very useful in the study of whole plant photophysiology. However, these mutants can also play an important role in the study of gene regulation. This has already been shown for the photoregulation of the gene for the chlorophyll *a/b* -binding protein (*cab*) in the *au* mutant. This study has demonstrated the usefulness of photomorphogenetic mutants and indicates their potential for future research.

SAMENVATTING

Planten gebruiken licht niet alleen als energie bron (fotosynthese), maar ook als informatie bron, waarbij groei en ontwikkeling afgestemd worden op het voor de plant beschikbare licht (fotomorfogenese). Er zijn een aantal fotoreceptoren, die het mogelijk maken informatie te verzamelen over het daglicht spectrum. Dit zijn: fytochroom (P), vnl. werkzaam in het rood (RL)/ver-rood (FR) spectrale gebied, blauw licht (BL)/UV-absorberende fotoreceptor(en) en praktisch uitsluitend in het UV absorberende UV-B fotoreceptor(en). Deze pigmenten kunnen kwaliteit, kwantiteit, richting en duur van het licht waarnemen en ontwikkelings processen hierop afstemmen. Veel processen in planten worden gereguleerd door deze fotoreceptoren, zoals kieming, lengte groei, enzym aktiviteit, fototropie en bloei.

Van deze fotoreceptoren is fytochroom het meest uitvoerig bestudeerd. Sinds kort is het bekend dat er meerdere types P bestaan: labiel (*l*P) en stabiel (*s*P) P. In het donker is voornamelijk *l*P aanwezig, terwijl in het licht *l*P en *s*P in vergelijkbare hoeveelheden voorkomen. Behalve dat er verschillende P types bestaan, zijn er ook nog verschillende werkingsmechanismen van P: de zeer lage intensiteit respons (very low fluence response: VLFR), lage intensiteit respons (low fluence response: LFR) en de hoge intensiteit respons (high irradiance response: HIR). De BL/UV-fotoreceptor(en) is/zijn nog hypothetisch. De aanwezigheid er van wordt afgeleid uit aktiespectra, maar de chemische samenstelling is nog onbekend. Ook de aard van de UV-B fotoreceptor(en) is nog niet opgehelderd. De fotoreceptoren werken in diverse processen samen. Het is echter nog niet duidelijk hoe deze samenwerking verloopt en wat de relatieve rol is van de verschillende fotoreceptoren.

De aanwezigheid van diverse fotoreceptoren met meerdere types en werkingsmechanismen maakt fotomorfogenese tot een zeer complex proces, vooral ook omdat vaak de absorptie spectra elkaar overlappen. In dit proefschrift wordt beschreven hoe mutanten, waarvan een deel van de fotomorfogenese ontbreekt, gebruikt kunnen worden in het fotomorfogenese onderzoek. Het belang van het ontbrekende deel wordt duidelijk door de mutant te vergelijken met het wild type. Deze mutanten kunnen, sterk vereenvoudigd, worden onderverdeeld in drie groepen: receptor-, transductie keten-, en respons mutanten (hoofdstuk 1). Er zijn nog slechts weinig fotomorfogenese mutanten bekend, enkele daarvan worden beschreven in hoofdstuk 2. De mutanten die voor het in dit proefschrift beschreven

onderzoek werden gebruikt zijn: de lange hypocotyl mutant (*lh*) van komkommer, en de *aurea* (*au*) en 'high pigment' (*hp*) mutanten van tomaat. Zowel de *lh* mutant als de *au* mutant worden gekenmerkt door een extra lange ('ge-etioleerde') hypocotyl als ze in wit licht (WL) worden opgekweekt. De *hp* mutant vertoont daarentegen een versterkte pigment synthese, vooral van anthocyaan.

Uit spectrofotometrische bepalingen bleek dat ge-etioleerde kiemplanten van de *au* mutant ten hoogste 5% (=detectiegrens) bevat van de hoeveelheid P aanwezig in het wild type (hoofdstuk 4). M.b.v. immunochemische technieken (uitgevoerd in samenwerking met de onderzoeksgroep van Prof. P.H. Quail (Plant Gene Expression Center, USDA, Albany, USA) werd dit bevestigd. Het bleek dat het P molecuul wel gevormd wordt, maar niet geaccumuleerd. Waarschijnlijk is het instabiel, maar het is echter nog niet duidelijk waarom. In in het licht opgegroeid weefsel waarin geen chlorofyl voorkomt (bloemblaadjes) of waarin dit is gebleekt (Norflurazon-behandelde bladeren) van de *au* mutant bleek de hoeveelheid spectrofotometrisch bepaalde P slechts 50% te zijn van dat in vergelijkbaar weefsel in het wild type. Hieruit werd geconcludeerd dat de *au* mutant een receptor mutant is en (bijna) geen *l*P bevat. Er zijn echter nauwkeurige immunochemische bepalingen nodig, waarbij m.b.v. antilichamen, specifiek tegen *s*P en *l*P, bovenstaande conclusie wordt geverifieerd. Deze antibodies zijn helaas nog niet geïsoleerd.

Het P gehalte van zaden en van ge-etioleerde kiemplanten van de *lh* mutant bleek gelijk te zijn aan dat van het wild type. In licht-gegroeid weefsel (bloemblaadjes en gebleekte bladeren) van de *lh* mutant werd spectrofotometrisch een P gehalte waargenomen dat ongeveer 50% is van de hoeveelheid in het wild type. Het is waarschijnlijk dat deze mutant geen *s*P bezit of anders een onwerkzame, spectrofotometrisch niet te detecteren, vorm ervan. Immunochemische technieken zullen ook in dit geval moeten uitwijzen of *s*P inderdaad afwezig is. Dit onderzoek wordt op het ogenblik uitgevoerd in samenwerking met de onderzoeksgroep van Prof. M. Furuya (Plant Biological Regulation, Frontier Research Programs (RIKEN), Saitama, Japan).

Met behulp van deze mutanten zijn verscheidene fotomorfogenetische processen bestudeerd. In hoofdstuk 3 wordt een beschrijving gegeven van in de literatuur beschreven fotofysiologische kenmerken van tomaat en komkommer. Bovendien worden enkele theorieën besproken in relatie tot de fotomorfogenese van deze beide soorten. In de daarop volgende hoofdstukken worden experimenten beschreven waarin deze kenmerken worden vergeleken bij de mutanten en hun wild types.

Onderzoek naar de invloed van licht op lange termijn groei van komkommer kiemplanten wordt beschreven in hoofdstuk 5. Hieruit blijkt dat ge-etioleerde kiemplanten van de *lh* mutant en het wild type bijna identiek reageren op de verschillende belichtingen. Na de-etiolatie treden er echter duidelijke verschillen op: de P controle van strekkingsgroei is sterk gereduceerd in de *lh* mutant. Bovendien is de de-etiolatie sterk vertraagd. In continu WL of RL wordt de hypocotyl van de *lh* mutant veel langer dan die van het wild type. Ook de (epidermis) cellen van de mutant zijn veel langer, vergelijkbaar met het verschil in hypocotyl lengte. Deze resultaten maken het aannemelijk dat het verschil tussen wild type en mutant ligt in de aan- respectievelijk afwezigheid van (funktioneel) *s*P.

Veel planten reageren op een FR puls aan het einde van de dag door extra lang te groeien. Ook komkommer vertoont deze respons. In de *lh* mutant is deze z.g. 'end-of-day FR' respons echter afwezig. Hieruit wordt geconcludeerd dat *s*P, dat ontbreekt of niet werkzaam is in de mutant, deze respons reguleert. Een andere respons, die waarschijnlijk ook door *s*P wordt gereguleerd, is de 'gesimuleerde' fototropie. Als één van de cotylen met aluminiumfolie wordt ingepakt en de plant vervolgens van boven wordt belicht met WL of RL, gaat de kiemplant krommen. Deze respons van ge-de-etioleerde kiemplanten wordt waarschijnlijk veroorzaakt door RL absorptie van P in de cotylen. Deze respons is echter afwezig in de *lh* mutant.

Zowel de hypocotyl strekking van het wild type als van de *lh* mutant worden door BL geremd. WL bevat echter ook BL en toch wordt de mutant lang in WL. Uit experimenten bleek dat de remming door BL werd veroorzaakt door een te lage fotosynthese als gevolg van de vrij lage, voor fotosynthese niet effectieve, lichtintensiteit. Bovendien is het mogelijk dat de remming o.i.v. een BL/UV-fotoreceptor slechts tijdelijk is. In WL is de fotosynthese hoog genoeg om te kunnen groeien. In tegenstelling tot de *lh* mutant wordt het wild type echter ook nog geremd door het deel van het licht dat door P wordt geabsorbeerd.

De lange hypocotyl van de *lh* mutant lijkt erg op die van een wild type behandeld met gibberelline (GA). Wild type en *lh* mutant reageerden echter in gelijke mate op een behandeling met GA. Bovendien treed het verschil in lengte alleen op in WL of RL en niet in BL of UV-A. Het effect van GA was echter vergelijkbaar in kiemplanten belicht met RL of BL. Dit wijst er op dat het effect van GA onafhankelijk was van de licht behandeling en geeft aan dat de *lh* mutant waarschijnlijk geen 'GA-overproducer' is. Er zijn echter op het ogenblik nog geen GA concentraties bepaald in de *lh* mutant of

het wild type.

Uit de literatuur blijkt dat er een duidelijk verschil is in de kinetiek van de hypocotyl remming door BL en RL. Om dit nerschil nauwkeurig(er) te kunnen meten is echter zeer gevoelige apparatuur nodig. Het gaat om veranderingen in groeisnelheden die binnen een minuut kunnen optreden. In eerste instantie is er een oriënterend onderzoek uitgevoerd m.b.v. een horizontale microscoop (hoofdstuk 6). Hieruit bleek al dat de remming door BL veel sneller optrad dan de remming door RL. Om het mogelijk te maken deze metingen zeer nauwkeurig en onder gecontroleerde omstandigheden uit te voeren aan zo veel mogelijk planten is er op het Laboratorium voor Plantenfysiologisch Onderzoek een apparaat ontwikkeld waarmee het mogelijk is groeiveranderingen waar te nemen binnen enkele seconden. Dit computer gestuurde apparaat wordt uitgebreid beschreven in hoofdstuk 6. De meetgegevens worden eveneens m.b.v. een computer verwerkt. Dit maakt het mogelijk grote hoeveelheden individuele planten met elkaar te vergelijken. Uit experimenten met deze continu-registrerende groeimeter bleek dat zowel ge-etioleerde kiemplanten van de *lh* mutant als van het wild type al na één minuut door BL werden geremd. De groeiremming door RL kon pas na enkele uren worden waargenomen. Er werden geen signifikante verschillen waargenomen tussen ge-etioleerde kiemplanten van de *lh* mutant en het wild type. Op grond van de resultaten van lange-termijn groeiproeven wordt echter verwacht dat er wel duidelijke verschillen zullen zijn bij ge-de-etioleerde kiemplanten. Hieraan zijn echter nog geen metingen met de groeimeter verricht.

Evenals de *lh* mutant van komkommer heeft ook de *au* mutant van tomaat een extra lange hypocotyl in WL. Bovendien is de kieming vertraagd, het chlorofylgehalte sterk verlaagd en kan er in ge-etioleerde planten niet meer dan 5% van de hoeveel P aanwezig in het wild type worden aangetoond. In hoofdstuk 4 wordt beschreven dat deze mutant waarschijnlijk geen *IP* bevat of in elk geval zeer weinig. In hoofdstuk 7 worden de resultaten beschreven van experimenten, opgezet om meer inzicht te krijgen in de rol die *IP* speelt in de fotomorfogenese. Hiertoe werd de respons van de *au* mutant in verscheidene fotomorfogenetische processen vergeleken met die van het wild type.

Ge-etioleerde kiemplanten van de *au* mutant bleken min of meer RL-blind te zijn. Dit wijst op een gebrek aan P controle in de mutant, kennelijk veroorzaakt door het gebrek aan *IP*. De hypocotyl strekking van de *au* mutant werd echter wel geremd door BL en UV-A. De *au* mutant bleek echter een hogere lichtintensiteit nodig te hebben om in dezelfde mate te worden geremd als

het wild type. Het wijst er op dat de 95% vermindering van de P concentratie in de *au* mutant dit verschil veroorzaakt. Hieruit wordt geconcludeerd dat dit in de *au* mutant afwezige *IP* een belangrijke rol speelt in de hypocotyl strekking van ge-etioleerde kiemplanten. Dit is de eerste keer dat er een duidelijke funktie is gevonden voor *IP*. Tot nu toe is het nooit gelukt een funktie te vinden van deze vorm van P, ondanks het feit dat *IP* in grote hoeveelheden aanwezig is in ge-etioleerde planten.

Twee andere experimenten met de *au* mutant versterken conclusies die werden getrokken naar aanleiding van experimenten met de *lh* mutant van komkommer. Zowel de 'end-of-day FR' respons als de 'gesimuleerde' fototropie werden in de *au* mutant waargenomen. Dit duidt op een regulatie van beide processen door *s*P.

Anthocyaan synthese is één van de processen die duidelijk door P worden gereguleerd. Er wordt verondersteld dat het niet mogelijk is om anthocyaan synthese te induceren in kiemplanten van tomaat met één enkele RL puls. Voordat deze inductieve P respons kan optreden moet het systeem eerst gevoelig gemaakt worden voor P door voorbelichting met BL of UV-A. Experimenten met de *hp* mutant van tomaat wijzen er echter op dat er wel anthocyaan wordt gevormd na één RL puls, maar dat dit te weinig is om te kunnen worden waargenomen (hoofdstuk 8). De *hp* mutant heeft een sterk verhoogde anthocyaan synthese, waardoor de respons op een RL puls wel kon worden waargenomen. De *au* mutant bevatte ook na een voorbelichting geen anthocyaan. Een dubbel mutant, geïsoleerd uit een kruising tussen *hp* en *au*, was echter wel in staat een meetbare hoeveelheid anthocyaan te synthetiseren. Deze hoeveelheid was ongeveer 3% van de hoeveelheid geproduceerd door de *hp* mutant. Dit wijst er op dat de 95% P die afwezig is o.i.v. de *au* mutatie een belangrijke rol speelt in de anthocyaan synthese. Naast de regulatie van hypocotyl strekking in ge-etioleerde kiemplanten is dit waarschijnlijk weer een funktie van *IP*.

In het laatste hoofdstuk worden enkele algemene conclusies en discussiepunten uiteengezet. Eén van die conclusies is dat Pfr de aktieve vorm van P is. Tot nu toe was nog niet echt bewezen wat het primaire aktieve fysiologische proces is in de P reaktie: de toename van de hoeveelheid Pfr of de afname van Pr. Het feit echter, dat mutanten met een gereduceerde hoeveelheid P (en dus ook minder Pr) in het donker even lang worden als het wild type, i.p.v. korter als gevolg van vermindering van Pr, wijst er echter op dat Pfr de aktieve vorm is.

Een andere conclusie is dat *IP*, in grote hoeveelheden voorkomend in ge-

etioleeerde planten, wel degelijk funktioneel is. Dit kon tot nu toe niet worden aangetoond. Uit een evolutionair oogpunt is dit ook wel waarschijnlijk. Het zou zeer 'oneconomisch' zijn voor een plant om een grote hoeveelheid niet funktioneel eiwit te maken in een ontwikkelingsstadium waarin alle energie nog uit reservevoorraden moet worden gehaald.

De mutanten die in dit proefschrift zijn beschreven blijken zeer geschikt om de fotofysiologie van hele planten te bestuderen. Echter, ook in de bestudering van gen regulatie kunnen deze mutanten een belangrijke rol spelen. Dit is gebleken uit het onderzoek naar de fotoregulatie van het gen voor het chlorofyl *a/b* -bindend eiwit (*cab*) in de *au* mutant. Dit onderzoek heeft de vele gebruiksmogelijkheden van fotomorfogenetische mutanten aangetoond en potentiële mogelijkheden aangegeven voor toekomstig onderzoek.

APPENDIX

Light sources

The light cabinets with broad-band light sources used in long-term growth experiments were qualitatively the same as those described by De Lint (1960) and Joustra (1970). The cabinets were situated in a dark rooms, kept at a temperature of 25 ± 0.5 °C and a relative humidity of about 65%.

Cabinet 'De Lint model':

The internal dimensions of these cabinets are: 60 cm wide, 100 cm high and 120 cm deep. They were equiped with fluorescent tubes or incandescent lamps and with glass plates and appropriate Plexiglas filters. The shelves were adjustable to enable variation of fluence rate. Separate cabinets were used for each light quality. The fluence rates used in particular experiments are described in the figure legends.

Cabinet 'Joustra model':

The internal dimensions of these cabinets are: 70 cm wide, 70 cm high and 110 cm deep. The walls of the cabinets are made of aluminium to optimize the light distribution. Light sources are mounted in a compartment on top of the cabinet. The lamp compartment provides space for 12 fluorescent tubes. These cabinets were used for irradiation with white (WL), red (RL) or blue (BL) light using the tubes and filters described below. The fluence rates used in particular experiments are described in the figure legends.

The fluence rate was measured with a photodiode meter (Optometer type 80X, United Detector Technology Inc., Santa Maria, CA, USA). The calibration of the Optometer was checked by comparison with a calibrated thermopile (Kipp en Zonen, Delft, The Netherlands). The spectral quality of the light sources was monitored with a Rofin-Sinar Spectralysor (Rofin-Sinar Laser UK Ltd., Weybridge) coupled with a microcomputer. The relative energy distributions are shown in Fig. 1. The values for [Pfr]/[Ptotal] (φ) were calculated on the basis of the molar absorption coefficients for Pfr and Pr from rye (kindly supplied by J.C. Lagarias) and quantum yield values as reported by Lagarias *et al.* (1987). A special computer program was developed for these calculations, utilizing the relative spectral energy distribution obtained with the light sources as monitored by the Rofin-Sinar Spectralysor and energy fluence rate measurements. The program also enabled determination

of (i) the minimum time necessary to obtain equilibrium of the P under a particular light source, (ii) the total photon fluence rate of a light source, (iii) the photon fluence rate of a particular part of the whole spectrum of a light source, and (iv) the cycling rate of the P photoconversion under a light source.

Specifications of the particular light qualities:

White light: fluorescent tubes type Philips TL40/33 with a transparent glass plate as a neutral filter.

UV-A light: Philips TL 40/08 with a transparent glass plate. Maximum emission (λ_{max}) at 359 nm; spectral half band width (HW) 38 nm. Contributions from individual mercury (Hg) lines to the total emitted energy were: 365 nm 0.8%; 404.7 + 407.7 nm 2.2%; 435.8 nm 0.35%. Fluence rate was modified using perforated aluminium sheets on top of the wooden boxes which contained the plastic boxes with seedlings.

Blue light: Philips TL40/18 with 3 mm Plexiglas blue 0248 (Röhm u. Haas). λ_{max} at 442 nm; HW 75 nm. Contribution from Hg lines were: 365 nm 0.07%; 404.7 + 407.7 nm 3.8%; 435.8 nm 17.7%; 546.1 nm 0.34%. Fluence rate was modified using additional 3 mm Plexiglas blue 0248 plates on top of the wooden boxes containing the plastic boxes which contained the seedlings.

Green light (GL): Philips TL40/17 with a sheet of yellow glass. λ_{max} at 527 nm; HW 42 nm. Contributions of the Hg lines were: 435.8 nm 0.09%; 546.1 nm 4.9%; 577 + 579 nm 1.02%.

Red light: Philips TL40/103339 with 3 mm Plexiglas red 501. λ_{max} at 658 nm. HW $\approx$ 18 nm, main peak 13 nm.

Far-red light (FR): Bank of 60 W 240 V incandescent lamps operated at 220 V with a layer of 10 cm running tap water and 3 mm Plexiglas red 501 and 3 mm blue 627. The energy of this light regime was calculated by subtracting from the total energy the fraction transmitted by 3 mm RG 780 (Schott u. Gen.) The resulting effective energy distribution is given in Fig. 1 as a broken line.

In short-term growth experiments, narrow-band BL (459 nm) and RL (658 nm) were obtained from a quartz-iodide projection lamp using interference filters (Balzer B40 type, Balzer Liechtenstein), 10 nm half-band width at 50% of the transmission maximum. The fluence rates used in particular

experiments are described in the figure legends.

Green safelight (fluence rate 40 nmol m^{-2} s^{-1}) was obtained by wrapping a Philips TL/33 fluorescent tube with 3 layers of No 39 Cinemoid (Rank Strand Electric, London).

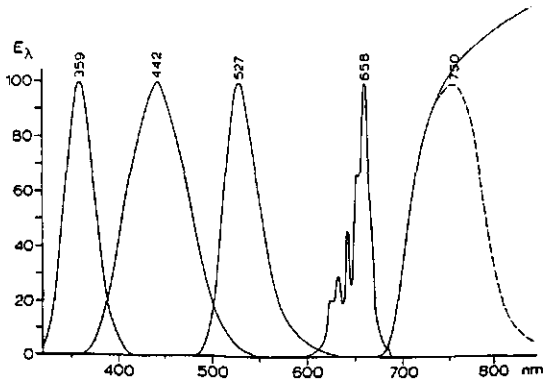

Fig. 1. Relative spectral energy distributions per nanometer of the broad band light sources used in this study. Mercury lines are not shown. (From Koornneef *et al.*, 1980).

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CURRICULUM VITAE

Ik ben geboren op 12 februari 1958 te Heerenveen. In 1976 behaalde ik het diploma Atheneum B op de R.S.G. Ooststellingwerf te Oosterwolde (Fr). Datzelfde jaar begon ik aan de studie biologie aan de Rijksuniversiteit te Groningen. Het kandidaatsexamen B1 behaalde ik in 1980 en het doctoraalexamen, met onderwijsbevoegdheid, in 1984. Hoofdvak: plantenfysiologie, bijvakken: cel- en plantengenetica, fysiologische microbiologie en milieukunde. In de jaren 1981, 1983 en 1984 was ik studentassistent bij resp. het 1^{e} jaars, 1^{e} fase en 2^{de} fase praktikum plantenfysiologie. Van 1 januari 1985 tot 1 januari 1988 was ik werkzaam als onderzoeksassistent bij de vakgroep Plantenfysiologisch Onderzoek van de Landbouwuniversiteit te Wageningen. Het onderzoek aan deze vakgroep, onder leiding van dr. R.E. Kendrick en prof. dr. W.J. Vredenberg, heeft geleid tot dit proefschrift. Vanaf 1 maart 1988 ben ik als plantecelbioloog in tijdelijke dienst bij het onderzoeksinstituut Ital te Wageningen, waar ik onderzoek doe naar de selectie van hoogproducerende cellijnen van Tagetes.

Paulien Adamse