# Genetic Analysis of Photomorphogenic Mutants in Tomato

Genetische analyse van fotomorfogenese mutanten in tomaat

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# STELLINGEN

**1.** De specifieke groeiwijze van de tomaat en de licht afwijkende rol van de tomaat fytochromen, maken de tomaat voor genetisch/fotofysiologisch onderzoek tot een belangrijke aanvulling op de modelsoort *Arabidopsis*.

**2.** Het extreme fenotype van fytochroom B deficiënte mutanten in *Arabidopsis*, *Brassica rapa* en komkommer heeft ten onrechte de indruk gewekt dat fytochroom B in alle plantensoorten het dominante stabiele fytochroom is.

**3.** Het ontbreken van een eind-van-de-dag verrood respons is niet altijd diagnostisch voor fytochroom B deficiëntie.

dit proefschrift

**4.** Het feit dat micro-injectie met fytochroom A het chlorofylgebrek van de *aurea* mutant kan opheffen, lijkt tegenstrijdig aan de waarneming dat fytochroom A deficiënte mutanten een normaal chlorofylgehalte hebben.

Neuhaus et al. *Cell* **73:** 937-952
dit proefschrift

**5.** Aangezien fotomorfogenetische processen door een aantal genen met op elkaar lijkende effecten gereguleerd worden, leidt een mutatie in slechts één van deze genen vaak tot een subtiel fenotype. Selectie in een mutante achtergrond kan de herkenbaarheid van deze subtiele fenotypen sterk verbeteren.

**6.** De toenemende neiging om het directe nut van wetenschappelijk onderzoek te willen meten, doet onrecht aan het lange termijn belang van wetenschappelijk onderzoek. Om zich tegen deze tendens te kunnen verweren, zou elke promovendus moeten beginnen met een cursus zelfverdediging.

**7.** Uit het feit dat schoolkinderen een half uur na de maaltijd opmerkelijk beter presteren en ouderen na de maaltijd graag een dutje doen, kan de conclusie getrokken worden dat promoties voortaan alleen nog plaats dienen te vinden om 13.30 uur.

De Volkskrant, 19-10-1996

**8.** Promovendi zouden op 6 december volgens oud Hollandse traditie hun promotie cadeau moeten krijgen.

**9.** Het beeld van de ideale wetenschapper neigt naar travestie: mannelijke voortvarendheid overgoten met vrouwelijke voorzichtigheid.

**10.** Vakkenvullen bij de Landbouwuniversiteit levert in tegenstelling tot bij de supermarkt financiële problemen op.

**11.** Bij het aardappelpitten slaat aardappelmoeheid toe zonder aaltjesoverdracht.

**12.** Voor Erasmusstudenten is het te hopen dat de draagkracht van deze wetenschappelijke brug bestand is tegen de stormen in het wetenschappelijk onderwijs.

**13.** Als een vrouw niet zingt, werkt zij ook niet veel.

Egyptisch spreekwoord

Stellingen behorende bij het proefschrift
"Genetic analysis of photomorphogenic mutants in tomato"

Wageningen, 6 december 1996 Ageeth van Tuinen

Ageeth van Tuinen



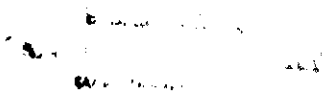
# Genetic Analysis of Photomorphogenic Mutants in Tomato

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 6 december 1996
des namiddags te half twee in de Aula.

Le terrain de la science est sûr, mais il ne représente qu'un îlot perdu dans l'océan illimité des choses inconnues.

Gustave le Bon

This research was supported by a grant from the Foundation for Life Sciences (SLW) formerly the Foundation for Biological Research (BION) which is subsidized by the Netherlands Organisation for Scientific Research (NWO).



CIP-gegevens Koninklijke Bibliotheek, Den Haag

Tuinen, A. van

Genetic analysis of photomorphogenic mutants in tomato / Ageeth van Tuinen. - [S.l.: s.n.] -I11.
Thesis Wageningen - With ref. - With summary in Dutch.
ISBN 90-5485-596-7
Subject headings: photomorphogenesis / phytochrome / *Lycopersicon esculentum*.

# Abstract

To sense light quality and quantity plants possess different groups of photoreceptors. This thesis describes the isolation and characterization of several photomorphogenic mutants in the model plant tomato. The use of a genetic approach to determine the roles of the different photoreceptors in photomorphogenesis and to study their mode of action, is one of the most promising approaches used in photomorphogenic research. $M_2$ populations were screened for aberrant seedling phenotypes in blue and/or red light (R). This resulted in the isolation of two allelic mutants (*fri*$^1$ and *fri*$^2$) that resembled the phytochrome A (phyA)-deficient mutants of *Arabidopsis* in their far-red light (FR) insensitivity. Immunochemically detectable phyA polypeptide is absent as is the bulk of the spectrophotometrically detectable labile phytochrome pool in etiolated *fri* seedlings. The *fri* locus mapped to chromosome 10 at a position similar to that of the *PhyA* locus on the RFLP map. Four allelic mutants, that had longer hypocotyls under continuous R, were isolated. These mutants are only insensitive to R during the first two days upon transition from darkness to R and were designated *tri*$^1$ to *tri*$^4$ (temporarily red light insensitive). Immunochemically detectable phyB1 protein is either absent, reduced or truncated. The *tri* mutants differ from phyB-deficient mutants found in other species in their relatively normal end-of-day FR and supplementary daytime FR responses. The *tri* mutant gene and the *PhyB1* gene were mapped to the same position on the RFLP map of chromosome 1. The nature of the mutations in *au* and *yg-2* was analysed by crossing both mutants with a transgenic tomato line that overexpresses oat phyA-3. Immunochemically detectable oat PHYA-3 is present in both the *au,PhyA-3* and *yg-2,PhyA-3* 'double mutant'. However, spectrophotometrical analysis revealed that it is non active, as indicated by the long-hypocotyl phenotype of the 'double' mutants. The results were consistent with both mutants being disturbed in the phytochrome chromophore biosynthesis. The three remaining phytochrome genes (*PhyB2*, *E* and *F*), for which no mutants have been found thus far, have also been mapped to the RFLP map of chromosomes 5, 2 and 7, respectively. In addition, it has been proved that there are two distinct *hp* loci, each with several alleles. The *hp-2* gene has been located on both the genetic and molecular linkage maps of chromosome 1.

# Contents


| | | |
|---|---|---|
| Chapter 1 | General introduction | 1 |
| Chapter 2 | Far-red light-insensitive, phytochrome A-deficient mutants of tomato | 19 |
| Chapter 3 | A temporarily red light-insensitive mutant of tomato lacks a light-stable, B-like phytochrome | 41 |
| Chapter 4 | Analysis of phytochrome-deficient *yellow-green-2* and *aurea* mutants of tomato | 63 |
| Chapter 5 | The mapping of phytochrome genes and photomorphogenic mutants of tomato | 85 |
| Chapter 6 | General discussion | 103 |
| References | | 113 |
| Samenvatting | | 125 |
| Nawoord | | 129 |
| Curiculum vitae | | 133 |

# Chapter 1

# General introduction

## Photomorphogenesis

Light plays a crucial role in the life cycle of plants. Plants not only need light as a source of energy, but also use it as a source of information. Depending on the light environment a different phenotype is displayed (Fig. 1). This influence on plant morphology by light is called photomorphogenesis and includes processes such as seed germination, de-etiolation, shade avoidance, and induction of flowering (Kendrick and Kronenberg 1994). De-etiolation results from the transition of a dark-adapted seedling (etiolated or exhibiting skotomorphogenesis), while below the soil surface, to a light-adapted green photosynthetic plant. This involves inhibition of hypocotyl growth, opening of the apical hook, expansion of the cotyledons, development of chloroplasts and in addition accumulation of anthocyanins in some species. Shade avoidance is the process by which plants respond to changes in the spectral quality of the light environment, caused by the proximity of other plants, enabling them to change their growth habit in an attempt to reach open sunlight. To sense the quantity, quality, duration and direction of light plants possess different types of photoreceptors.

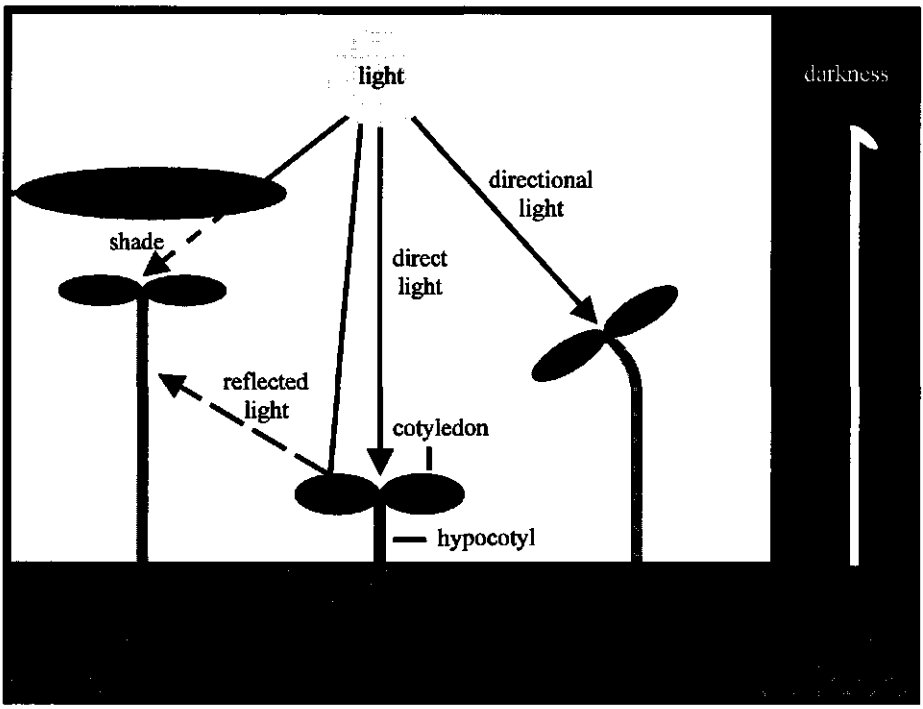



**Figure 1.** Schematic presentation of photomorphogenic developmental patterns of tomato seedlings grown under various white light environments. After McNellis and Deng (1995).

# Photoreceptors

## Phytochrome

The photoreceptor phytochrome exists in two forms: a red light (R)-absorbing (Pr) and a far-red light (FR)-absorbing (Pfr) form. Absorption of R converts Pr to physiologically active Pfr. This reaction is reversible. Absorption of FR by Pfr reverts it back to inactive Pr. The phytochrome molecule exists as a homodimer. Each monomer consists of an apoprotein to which a tetrapyrrole chromophore is covalently attached (Terry et al. 1993). In higher

plants the apoproteins are encoded by a small gene family (Pratt 1995; Quail 1994). However, it is assumed that all apoproteins carry the same chromophore (Rüdiger and Thümmler 1994; Terry et al. 1993). Phytochromes can be classified into two types: a light-labile (or type I) type, in which Pfr is rapidly degraded after formation by irradiation and a relatively light-stable (or type II) type. The first is represented by phytochrome A (phyA), whereas all other phytochromes determined so far seem to belong to the second type (Quail 1994). The expression level of the various phytochrome genes in tomato differs quantitatively between genes, per gene throughout the plant life cycle, and in spatial distribution (Hauser et al. 1994; Pratt 1995).

*Phytochrome mediated responses*

In addition to multiple phytochromes, the phytochrome system is even more complex due to the different modes of action. These modes are classified according to the quantity of light required: very low fluence responses (VLFR), low fluence responses (LFR) and high irradiance responses (HIR). Saturation of VLFRs is reached at extremely low fluences ($10^{-4}$ - $10^{-1}$ $\mu$mol $m^{-2}$) and Pfr concentrations. The VFLRs are not R/FR reversible, since even FR can elicit enough Pfr to induce the response itself. The LFRs are the classical R/FR reversible responses, which can be induced by a short pulse of R (1 - 1000 $\mu$mol $m^{-2}$). The LFR obeys the reciprocity law, *i.e.* a response is independent of irradiation time or fluence rate ($\mu$mol $m^{-2}$ $s^{-1}$) at a given total fluence ($\mu$mol $m^{-2}$). The HIRs require continuous irradiation. The extent of the HIR depends upon wavelength, irradiance and duration of the light treatment. The HIRs are not R/FR reversible and the reciprocity law does not hold. While the exact mechanism is not understood, the response is related to the photon irradiance and the plant somehow counts photons, which is reflected by the parameter phytochrome cycling rate (the rate of interconversion of phytochrome between its two forms). The existence of a light-labile (type I) phytochrome explains why FR is more effective than R, since FR maintains a higher integrated phytochrome level for cycling in such a case. For stable phytochromes R is more effective than FR, because it

establishes a higher Pfr/Ptot ratio (where Ptot = Pr + Pfr) and more efficiently cycles phytochrome (Mancinelli 1994).

In addition, responses, exhibited by fully de-etiolated plants, have been identified, such as: photoperiodic, end-of-day and R:FR photon ratio perception responses. Photoperiodic responses are responses to the duration of the light and dark periods in a 24 h cycle and include time keeping mechanisms. The role of phytochrome in this is not fully understood (Vince-Prue 1994). End-of-day responses are effects of the light-environment on the state of phytochrome at the end of the daily light period and therefore on the phytochrome status in the following dark period. The responses are R/FR reversible and obey the reciprocity law. The R:FR ratio perception responses are the basis of the shade avoidance response (Fig. 1): an increase in stem growth as a result of decreasing R:FR ratios due to absorption of R and transmission of FR by shading vegetation (Smith 1995). Both R:FR ratio perception and end-of-day responses are thought to have a common underlying mechanism.

*Phytochrome signal transduction*

Although, phytochrome was discovered more than 50 years ago, its mode of action remains unclear. The induction of the expression of particular genes by light can be considered as the end point of a signal transduction chain. Recently evidence has been provided for G-protein and calcium mediated signal transduction, as found in many animal signal transduction chains.

Heterotrimeric G-proteins have been suggested to initiate the cascade of events of the phytochrome signal transduction pathway (reviewed by Miller et al. 1994), although this still has to be proved by purification and characterization of the G-protein(s). It also has to be clarified as to whether the putative plant G-protein(s) resembles its animal counterpart in its subcellular localization as a membrane bound protein (Miller et al. 1994). Assuming this is the case, phytochrome, being a soluble cytosolic protein, has either to migrate to the membranes to activate the G-proteins or use an intermediate to relay the signal.

Microinjection of purified oat phyA into hypocotyl cells of the phytochrome deficient *aurea* mutant of tomato, initiated chloroplast development, chlorophyll *a/b*-binding protein gene expression and anthocyanin production (Neuhaus et al. 1993). Further research by Bowler et al. (1994), using the same methodology, revealed that $Ca^{2+}$/calmodulin and cyclicGMP appeared to act as transducers downstream of phyA and G-proteins, in both parallel and converging pathways, to regulate the above mentioned processes (Fig. 2). The mechanism by which the G-proteins mobilize $Ca^{2+}$ has yet to be determined and an answer to the question, whether other phytochromes can share the phyA signal transduction pathway or have their own separate pathways, has to be found.

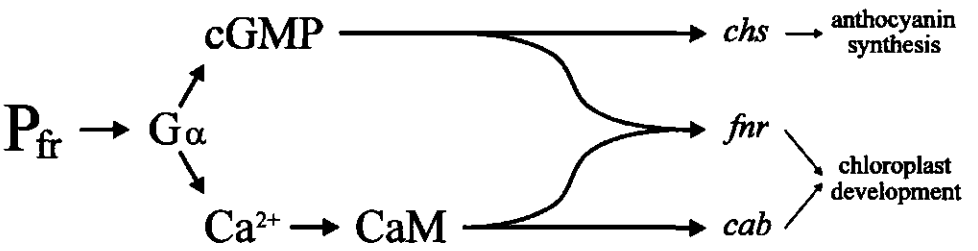



**Figure 2.** Biochemical model of PHYA signal transduction. $P_{fr}$ activation of the α subunit of heterotrimeric G proteins (Gα) leads, via cyclic GMP (cGMP) and $Ca^{2+}$/Calmodulin ($Ca^{2+}$/CaM) dependent pathways, to expression of chalcon synthese (*chs*), ferrodoxin $NADP^+$ oxireductase (*fnr*) and chlorophyll *a/b* binding protein (*cab*) genes, resulting in anthocyanin synthesis and chloroplast development. After Bowler et al. (1994).

## Blue- and UV-A light-absorbing photoreceptors

Although, phytochrome also absorbs in the blue (B) and UV-A region of the spectrum, it is generally accepted that there are additional B and UV-A photoreceptors (Mohr 1994).

Physiological arguments for specific B receptors came from B and UV effects on processes such as phototropism and their effects on for instance growth inhibition and pigment biosynthesis, that could not be explained by absorption of these wavelength by phytochrome (Jenkins 1995; Senger and Schmidt 1994).

The nature of one B receptor was elucidated by cloning the *HY4* gene in *Arabidopsis* (Ahmad and Cashmore 1993, 1996). When mutated this gene leads to a partially defective B response (Koornneef et al. 1980). The *HY4* gene encodes a protein, called cryptochrome 1 (CRY1), which has homology to microbial DNA photolyases. These flavoproteins catalyze B-dependent repair of DNA and have both flavin and pterin chromophores (Senger and Schmidt 1994). *In-vitro* experiments revealed that the CRY1 protein is able to bind both flavins and pterins (Lin et al. 1995b; Malhotra et al. 1995). Overexpression of the CRY1 protein in transgenic tobacco plants proved that the CRY1 protein functions as a B/UV-A photoreceptor (Lin et al. 1995a).

Phototropism, which is a specific B response, appears to be encoded by a photoreceptor different from cryptochrome 1 (Jenkins et al. 1995). Reymond et al. (1992) showed that B stimulates phosphorylation of a non-phosphorylated membrane protein, which is severely reduced in the *nph1* mutants. These mutants lack the phototropic response, but hypocotyl elongation is still inhibited by B. The NPH1 protein is presumed to be the non-phosphorylated membrane protein and the photoreceptor responsible for phototropic responses in *Arabidopsis* (Liscum and Briggs 1995). Based on physiological evidence and the observation that the *hy4* mutant responds normally to other B responses, such as stomatal opening and chloroplast development, the presence of additional B and UV-A receptors (Jenkins et al. 1995) is obvious.

## UV-B

Most of the physiological effects of UV-B cause damage, in particular to

DNA. Plants can either use shielding or repair mechanisms to limit the harmful effects of UV-B. Exposure to UV-B induces gene expression of genes encoding components related to flavonoid biosynthesis, leading to increased levels of UV-absorbing pigments, including anthocyanins. In *Arabidopsis*, mutants that are disturbed in DNA repair or flavonoid biosynthesis have been found. However, little is known about UV-B photoreceptors or their signal transduction (Jenkins et al. 1995).

## Interaction between photoreceptors

Both phytochrome and B photoreceptors play a role in the control of processes such as hypocotyl elongation and anthocyanin synthesis (Mancinelli 1994). The mechanism by which the photoreceptors coact is complicated by the fact that B/UV-A activates phytochrome (Mohr 1994). Two hypothesis for coaction have been postulated by Mohr (1994): an independent coaction, by which both photoreceptors independently cause the same terminal response, and an interdependent coaction, by which the photoreceptors depend upon each other to produce the final response.

Experiments by Mohr and co-workers (Mohr 1994) favour the hypothesis that B and UV-A lead to an increased responsiveness to phytochrome, which is the actual effector of the de-etiolation response. A similar conclusion was reached for the action of CRY1 in B (Ahmad and Cashmore 1996).

## Photomorphogenic mutants

To understand the complex process of photomorphogenesis, it is necessary to find out what are the specific roles of the various phytochromes, the B/UV-A photoreceptors, the UV-B photoreceptor(s) and their mechanisms of coaction.

In the last decades the genetic approach, i.e. the search for mutants deficient in one of the photoreceptors or regulatory mutants, which display a distorted photomorphogenesis, has proved successful in dissecting the complexities of photomorphogenesis.

The initial screens for photomorphogenic mutants, which led to the identification of the first photoreceptor mutants in *Arabidopsis* (Koornneef et al. 1980), were performed in white light (WL). However, in later years, broad-band B, R and FR were used to select mutants deficient in photoreceptors, which detect discrete spectral regions, or defective in their transduction chain(s). Transduction chain mutants for a photoreceptor should have the same pleiotropic phenotype as the photoreceptor mutant, but in contrast to the latter should possess a photoreceptor that is still active *in vivo* (Koornneef and Kendrick 1994; Whitelam and Harberd 1994).

Another way of finding out the roles in photomorphogenesis specific to one type of photoreceptor, providing the gene for the photoreceptor has been cloned, is to use transgenic plants that over- or underexpress that particular photoreceptor. Transgenic plants overexpressing deletion derivatives of phytochrome A and B genes have been very useful in identifying the $NH_2$-terminal chromophore bearing domain as necessary for photosensory specificity and the $COOH_2$-terminal domain for regulatory activity. Photochemically active, missense mutants for phyA and phyB were selected. Sequence analysis revealed that 76% of the mutations resided in the same region of the COOH-terminal domain. The observation that regulatory activity of the photoreceptors requires COOH-terminal domain sequences common to both phyA and phyB is proved by the fact that one mutation was detected in both phyA and phyB (Quail et al. 1995; Wagner et al. 1996).

## Photoreceptor mutants

### *Phytochrome*

Phytochrome proteins are encoded by a small number of genes. In *Arabidopsis*, for instance, five phytochrome genes (*A-E*) have been isolated (Quail

1994). To unravel the roles of the different phytochrome species in photomorphogenesis, mutants deficient in specific phytochromes are essential tools. Three classes of phytochrome photoreceptor mutants have been identified so far. Chromophore mutants, which are deficient in the biosynthesis of the chromophore, and therefore deficient in all types of phytochrome, have been found in *Arabidopsis* (Koornneef et al. 1980; Parks and Quail 1991), *Nicotiana plumbaginifolia* (Kraepiel et al. 1994) and also recently in pea (Weller et al. 1996). Phytochrome A-deficient mutants have been described for *Arabidopsis* (Dehesh et al. 1993; Nagatani et al. 1993; Parks and Quail 1993; Whitelam et al. 1993). Phytochrome B-deficient mutants have been reported in several species including *Arabidopsis* (Reed et al. 1993), *Brassica rapa* (Devlin et al. 1992), cucumber (López-Juez et al. 1992) and pea (Weller et al. 1995).

The phenotype, for seedlings growing in continuous light, of these three mutant classes in all species analyzed is: phyA-deficient mutants are insensitive to FR; phyB-deficient mutants are insensitive to R, and phytochrome chromophore mutants are insensitive to both R and FR. The identification of these mutants has enabled specific responses to be attributed to different phytochromes. The FR-HIR for hypocotyl growth inhibition was assigned to phyA, whereas the effectiveness of R in inhibition appeared to be regulated mainly by phyB. Since the phyB-deficient mutants fail to respond to EODFR and daytime supplemental FR, it was proposed that phyB is also involved in the classic R/FR reversible LFRs (Smith 1995).

Little is known about mutants for the other type II phytochromes (C-E). It is therefore not clear if these are simply redundant phytochromes, with a function similar to phyB, that have no specific phenotype when mutated, because phyB can supplement for their loss. However, it is not excluded that their physiological role depends on their specific expression pattern or that they have specific functions and their absence causes only subtle phenotypes. That the absence of one of the type II phytochromes can cause only a subtle phenotype was recently proved by the fact that the *Arabidopsis* Ws ecotype was found to carry a naturally-occurring deletion

allele of the phytochrome D gene and was shown to lack the PHYD apoprotein. PhyD seems to regulate several of the same R responses as phyB, which is indicated by the additive phenotype of a double mutant lacking both phyB and phyD (Sharrock 1996).

*B/UV-A*

*Arabidopsis* is the first and so far only species for which a mutant (*hy4*) has been isolated, in which hypocotyl elongation is inhibited to the same extent as wild type (WT) in R and FR, but is less inhibited in B. Spectrophotometric (Koornneef et al. 1980) and immunochemical analysis (Somers et al. 1991) revealed that the phytochrome content did not differ from WT. The *HY4* gene has been cloned and encodes the CRY1 protein (Ahmad and Cashmore 1993), which is identified as a B photoreceptor (Lin et al. 1995a). A search for phototropic mutants resulted in the isolation of the *nph* mutants (Khurana and Poff 1989; Liscum and Briggs 1995) and provided substantial evidence for a specific B photoreceptor, controlling phototropism (Jenkins et al. 1995; Liscum et al. 1992). The fact that a *hy4,hy1* double mutant, in contrast to double mutants lacking both phytochrome A and B, still responds to B is explained by the trace of phytochromes present due to leakiness of the *hy1* mutant (Ahmad and Cashmore 1996; Koornneef et al. 1980; Young et al. 1992). However, in UV-A the hypocotyl elongation of the *hy4hy1* double mutant is inhibited to a greater extent than in B (Koornneef et al. 1980; Young et al. 1992), suggesting that another photoreceptor is involved in the UV-A controlled hypocotyl elongation. Data on hypocotyl elongation in UV-A of triple mutants lacking the PHYA, PHYB and CRY1 proteins are eagerly awaited to support this hypothesis.

*Regulatory mutants*

Mutants defective in signal transduction are needed to enlighten the black box of events from light perception to final response. Mutants deficient in signal transduction of a photoreceptor would be expected to have a similar phenotype to that of photoreceptor-deficient mutants for the photoreceptor

concerned, but they should still contain the active photoreceptor. Thus far, the *Arabidopsis hy5* (Koornneef et al. 1980) and *fhy1-1* (Whitelam et al. 1993) mutants are candidates of this type. The *hy5* mutant has a long hypocotyl in B, UV-A, R and FR, indicating that HY5 is necessary for modulating responses to phytochrome and the B/UV-A photoreceptors (Koornneef et al. 1980). The *fhy1-1* mutant displays an elongated phenotype in FR, but not in R and WL and in this resembles the phyA-deficient mutants. However, it has normal levels of spectrophotometrically and immunochemically detectable phyA (Whitelam et al. 1993). Johnson et al. (1994) showed that the *fhy1-1* mutant is affected in some, though not all, phyA mediated responses, indicating that the phyA signal transduction pathway has at least two branches and that FHY1 is involved with one of these branches.

Several research groups have searched for mutants exhibiting light-regulated responses in the absence of light. This has resulted in the isolation of the *det* (Chory et al. 1989, 1991) and *cop* (Deng and Quail 1992, Hou et al. 1993, Wei and Deng 1992) mutants of *Arabidopsis*. Recently, it has been shown that some *det* and *cop* mutants are allelic to the previously isolated *fus* mutants (Misèra et al. 1994). It is suggested that the DET/COP/FUS gene products are required to repress photomorphogenic development in darkness. The pleiotropic effects of the mutations imply that the DET/COP/FUS gene products act before branching of the pathway leading to specific light-regulated responses (McNellis and Deng 1995). One possibility is that the proteins function in close proximity with each other in the same pathway, for instance by the formation of multisubunit protein complexes. This seems to hold true for the COP9, COP8 and COP11 proteins (Wei et al. 1994a). Alternatively, the loci might define multiple parallel pathways that control the developmental switch from skotomorphogenesis to photomorphogenesis (Chory 1993; Misèra et al. 1994). Mutants of the *DET2* locus display a photomorphogenic morphology in darkness, but do not develop chloroplasts (Chory et al. 1991), thus demonstrating that chloroplast development is separable from other aspects of photomorphogenesis. Recently, it was found

that the *det2* mutant phenotype in dark, as well as in light, can be restored to WT by low concentrations of brassinosteroids. The DET2 protein probably encodes a reductase involved in brassinosteroid biosynthesis (Li et al. 1996). Light might act by modulating this hormone-like signal transduction pathway by regulating the biosynthesis of the brassinosteroids or by altering the responsiveness of the cells to brassinosteriods.

Based upon genetic interaction between *hy5* and *cop* mutants (Wei et al. 1994a, 1994b) the current hypothesis is that signals from phytochrome and other photoreceptors converge at or before HY5, probably upstream of the COP/DET/FUS complex. The HY5 protein might be involved in the control of COP1 nuclear localization and/or activity of the formation of the COP9 complex (Ang and Deng 1994).

In tomato yet another class of regulatory mutants is represented by the *hp* mutant, which exhibits exaggerated phytochrome responses, although no difference in the phytochrome content compared to WT has yet been found (Adamse et al. 1989; Peters et al. 1989, 1992). In contrast to the *cop/det/fus* mutants this mutant resembles WT in darkness and only shows an exaggerated response in the light. Normal photomorphogenesis of the tomato seedling requires coaction of the B receptor and phytochrome (Oelmüller and Kendrick 1991), a prerequisite which is lost in the *hp* mutant. Peters et al. (1992) proposed that the HP gene product inhibits the amplification of the phytochrome response, whereas this inhibiting effect can be overcome by B.

## Scope of the thesis

For the research here tomato has been chosen as a model system. Tomato has the advantage of being easier to handle than *Arabidopsis* in physiological experiments concerning hypocotyl elongation and anthocyanin content. The genetic and molecular (RFLP) marker maps are well defined and provide a solid basis for positional cloning strategies.

The tomato distinguishes itself by the fact that the phytochrome gene family comprises at least 9 and probably as many as 13 genes (Pratt 1995). Five of these genes are characterized and their expression patterns determined (Hauser et al. 1995; Pratt 1995; Pratt et al. 1995). It is obvious that elucidation of the pathways leading to photomorphogenic responses in tomato requires identification and characterization of specific photomorphogenic mutants.

At the start of this research project only a few photomorphogenic mutants of tomato were available. The *au* mutant has been used extensively in an attempt to understand the nature of the lesions (Parks et al. 1987; Sharrock et al. 1988), to study its photophysiology (Adamse et al. 1988) and more recently to investigate phytochrome signal transduction (Bowler et al. 1994). However, due to contradictory results it was not clear as to whether this was a specific phyA-deficient or a chromophore mutant. This was also the case for the *yg-2* mutant, which resembles the *au* mutant in its phenotype (Koornneef et al. 1985).

The second category of mutants available is illustrated by the *hp* mutant, which had been characterized as having an exaggerated phytochrome response (Adamse et al. 1989; Peters et al. 1989). Due to conflicting literature about allelism tests, it was not clear whether there were two *hp* loci or only one locus having two alleles (Mochizuki and Kamimura 1986; Soressi 1975).

This thesis describes the isolation and characterization of new photomorphogenic mutants (Chapters 2 and 3) and further analysis of the *au* and *yg-2* mutants (Chapter 4). The five characterized phytochrome genes, the new mutants and the *hp-2* locus were mapped to the genetic and/or molecular marker maps (Chapter 5). The nature of the various available and putative photomorphogenic mutants in tomato is discussed, as are the perspectives to use these mutants in understanding photomorphogenesis (Chapter 6).

## Account

Chapters 2-5 of this thesis have already been published or are in press. These and other publications, arising from or related to this study, in which I am an author, are given below.

Adamse P, Peters JL, Jaspers PAPM, **Van Tuinen A**, Koornneef M, Kendrick RE (1989) Photocontrol of anthocyanin synthesis in tomato seedlings: a genetic approach. *Photochem Photobiol* **50:** 107-111

Peters JL, **Van Tuinen A**, Adamse P, Kendrick RE, Koornneef M (1989) High pigment mutants of tomato exhibit high sensitivity for phytochrome action. *J Plant Physiol* **134:** 661-666

Peters JL, Wesselius JC, Georghiou KC, Kendrick RE, **Van Tuinen A**, Koornneef M (1991) The physiology of photomorphogenetic tomato mutants. In: *Phytochrome properties and biological action.* Thomas B, Johnson CB (eds), Springer Verlag, Heidelberg, pp 237-247

Koornneef M, **Van Tuinen A**, Kerckhoffs LHJ, Peters JL, Kendrick RE (1992) Photomorphogenetic mutants of higher plants. In: *Progress in Plant Growth Regulation. Proceedings 14$^{th}$ International Conference on Plant Growth Substances*. Karssen CM, Van Loon LC, Vreugdenhil D (eds), Kluwer Academic Publishers, Dordrecht, pp 54-64

Kendrick RE, Kerckhoffs LHJ, Pundness AS, **Van Tuinen A**, Koornneef M, Nagatani A, Terry MJ, Tretyn A, Cordonnier-Pratt M-M, Hauser B, Pratt LH (1994) Photomorphogenic mutants of tomato. *Euphytica* **79:** 227-234

Kendrick RE, Peters JL, Kerckhoffs LHJ, **Van Tuinen A**, Koornneef M (1994) Photomorphogenic mutants of tomato. In: *Molecular Botany: Signals and the Environment.* Bowles DJ, Gilmartin PM, Knox JP, Lunt GG (eds), Portland Press, London, pp 249-255

**Van Tuinen A**, Kerckhoffs LHJ, Nagatani A, Kendrick RE, Koornneef M (1995) Far-red light-insensitive, phytochrome A-deficient mutants of tomato. *Mol Gen Genet* **246:** 133-141

**Van Tuinen A**, Kerckhoffs LHJ, Nagatani A, Kendrick RE, Koornneef M (1995) A temporarily red light-insensitive mutant of tomato lacks a light-stable, B-like phytochrome. *Plant Physiol* **108:** 939-947

**Van Tuinen A,** Hanhart CJ, Kerckhoffs LHJ, Nagatani A, Boylan MT, Quail PH, Kendrick RE, Koornneef M (1996) Analysis of phytochrome-deficient *yellow-green-2* and *aurea* mutants of tomato. *Plant J* **9:** 173-182

Kerckhoffs LHJ, **Van Tuinen A,** Hauser B, Cordonnier-Pratt M-M, Nagatani A, Koornneef M, Pratt LH, Kendrick RE (1996) Molecular analysis of *tri*-mutant alleles in tomato indicates the *tri* locus is the gene encoding the apoprotein of phytochrome B1. *Planta* **199:** 152-157

**Van Tuinen A,** Cordonnier-Pratt M-M, Pratt LH, Verkerk R, Zabel P, Koornneef M The mapping of phytochrome genes and photomorphogenic mutants of tomato. *Theor Appl Genet* (in press)

Kerckhoffs LHJ, De Groot NAMA, **Van Tuinen A**, Schreuder MEL, Nagatani A, Koornneef M, Kendrick RE Physiological characterization of exaggerated-photoresponse mutants of tomato. *J Plant Physiol* (in press)

Kerckhoffs LHJ, Schreuder MEL, **Van Tuinen A**, Koornneef M, Kendrick RE Phytochrome control of anthocyanin biosynthesis in tomato seedlings: analysis using photomorphogenic mutants. *Photochem Photobiol* (in press)

# Chapter 2

# Far-red light-insensitive, phytochrome A-deficient mutants of tomato

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Published in *Mol Gen Genet* (1995) **246:** 133-141

**Abstract** *We have selected two recessive mutants of tomato with slightly longer hypocotyls than the wild type, one under low fluence rate (3 μmol $m^{-2}$ $s^{-1}$) red light (R) and the other under low fluence rate blue light. These two mutants were shown to be allelic and further analysis revealed that hypocotyl growth was totally insensitive to far-red light (FR). We propose the gene symbol* fri *(far-red light insensitive) for this locus and have mapped it on chromosome 10. Immunochemically detectable phytochrome A polypeptide is essentially absent in the* fri *mutants as is the bulk spectrophotometrically detectable labile phytochrome pool in etiolated seedlings. A phytochrome B-like polypeptide is present in normal amounts and a small stable phytochrome pool can be readily detected by spectrophotometry in the* fri *mutants. Inhibition of hypocotyl growth by a R pulse given every 4 h is quantitatively similar in the* fri *mutants and wild type and the effect is to a large extent reversible if R pulses are followed immediately by a FR pulse. After 7 days in darkness, both* fri *mutants and the wild type become green on transfer to white light, but after 7 days in FR, the wild-type seedlings that have expanded their cotyledons lose their capacity to green in white light, while the* fri *mutants de-etiolate. Adult plants of the* fri *mutants show retarded growth and are prone to wilting, but exhibit a normal elongation response to FR given at the end of the daily photoperiod. The inhibition of seed germination by continuous FR exhibited by the wild type is normal in the* fri *mutants. It is proposed that these* fri *mutants are putative phytochrome A mutants which have normal pools of other phytochromes.*

# Introduction

The control of plant development by light involves different groups of photoreceptors: those that absorb in the blue light (B), UV-A, UV-B, and the red light (R)/far-red light (FR)-absorbing phytochromes. Together they control processes of seedling development such as inhibition of hypocotyl growth, opening of the apical hook, expansion of the cotyledons and the accumulation of anthocyanin. Mutants deficient in one of these photorecep-

tors can be used to reveal the contribution of the various photoreceptors and their interaction in the regulation of photomorphogenic responses.

In *Arabidopsis thaliana*, the isolation and characterization of the phytochrome-deficient mutants (reviewed by Koornneef and Kendrick 1994), the *blu* mutants (Liscum and Hangarter 1991), and the evidence that the *HY4* gene encodes a protein with characteristics of a B photoreceptor (Ahmad and Cashmore 1993), proves that mutants for different photoreceptors can be isolated and are useful tools for dissection of the complex network of light-regulated responses in photomorphogenesis. Furthermore, Young et al. (1992) using the *blu* and *hy6* mutants and the *blu,hy6* double mutant found that the UV-A photosensory system caused inhibition of hypocotyl elongation by a process independent of the phytochrome and B photosensory systems. The fact that phototropism and B inhibition of hypocotyl elongation are genetically separable (Liscum et al. 1992) indicates that these processes may be controlled by different B photoreceptors. Not only the B photoreceptor system but also the R/FR-absorbing phytochrome system is more complex than initially thought. For instance, in *Arabidopsis* the phytochrome family consists of at least five different genes referred to as *PHYA-E*, which encode apophytochrome PHYA-E, and form holophytochrome phyA-E, respectively, after insertion of the chromophore (Quail 1994). To study the role of each phytochrome species in photomorphogenesis, mutants are needed which are deficient in one specific type of phytochrome. So far, three types of phytochrome mutants have been characterized. Mutants deficient in phyB have been reported in several species, including *Arabidopsis* (Reed et al. 1993), cucumber (López-Juez et al. 1992) and *Brassica rapa* (Devlin et al. 1992), whereas mutants lacking phyA have only more recently been characterized in *Arabidopsis* (Dehesh et al. 1993; Nagatani et al. 1993; Parks and Quail 1993; Whitelam et al. 1993). The third type of mutant, represented by the *hy1* and *hy2* mutants of *Arabidopsis* (Parks and Quail 1991) and the recently described *pew* mutants of *Nicotiana plumbaginifolia* (Kraepiel et al. 1994), are apparently deficient in the biosynthesis of the phytochrome chromophore.

In tomato, which is an attractive model system because of its favourable size for physiological experiments, the extensively studied *aurea* mutant has been considered a phyA-deficient mutant. However, there are indications that this mutant might be a chromophore mutant (Sharma et al. 1993; A. van Tuinen unpublished data). Since all phytochromes share the same chromophore, a chromophore mutant will presumably be deficient in all types of phytochrome. In an endeavour to search for type-specific phytochrome mutants in tomato, we have screened for mutants at different wavelengths in an attempt to avoid escape of mutants with minor phenotypic effects in white light (WL). In this paper we present the isolation and characterization of a mutant deficient in immunologically and spectrophotometrically active phyA.

## Results and discussion

### Mutant isolation and genetic characterization

The $M_2$ populations of tomato derived from EMS-treated seeds were screened under continuous B and R. Two independently induced mutants, 1-7RL and 1-17BL, were selected for their slightly longer hypocotyls in R and B, respectively. In a broad-band spectral scan both mutants, unlike the wild type (WT) were shown to be completely insensitive to FR. We therefore propose the gene symbol *fri* (far-red insensitive) for these mutants. Genetic complementation analysis showed that the two mutants were allelic. The 1-7RL is more extreme than the 1-17BL and we refer to them as $fri^1$ and $fri^2$, respectively. Under continuous FR the progeny of selfed $F_1$ plants from the cross between the new mutant lines and the WT parent segregated in a 3:1 ($\chi^2 = 3.59$, $P > 0.05$) ratio of normal to elongated hypocotyls expected for a monogenic recessive mutation. Data for $fri^2$ are given in Fig. 1.

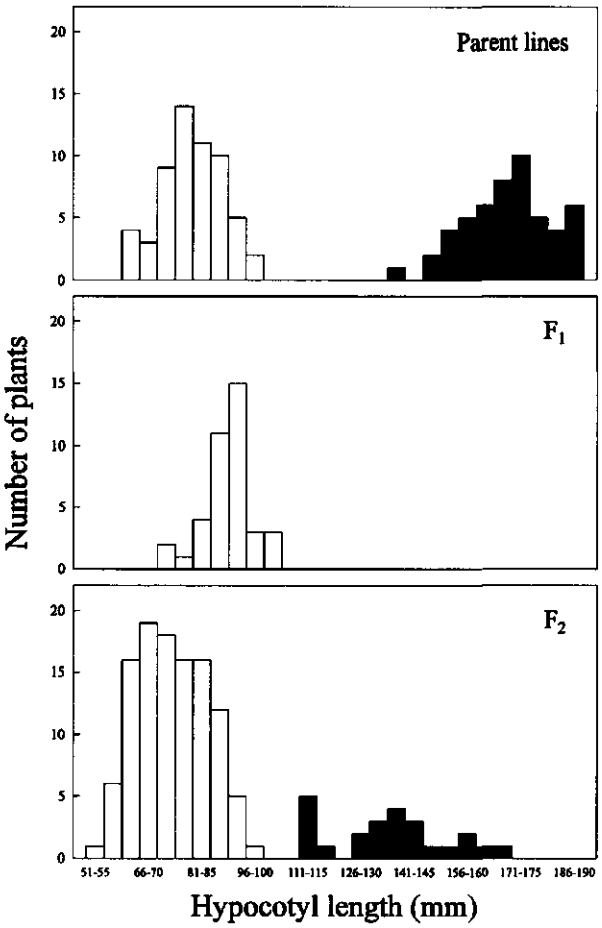


**Figure 1.** Frequency distribution of hypocotyl length of tomato seedlings of the wild type (cv. MM; *open bars*), *fri*$^2$ mutant (*filled bars*), $F_1$ and $F_2$ generations after 7 days of continuous far-red light (3 μmol $m^{-2}$ $s^{-1}$).

## Phenotypes of the *fri*$^1$ and *fri*$^2$ mutants

When grown in FR, plants homozygous for the *fri*$^1$ and *fri*$^2$ mutations do not differ from plants grown in D. The hypocotyls are elongated, the apical hooks closed and the cotyledons are unexpanded. The WT plants grown in FR exhibit less hypocotyl growth inhibition than in B and R, and the cotyledons remain yellow, but are fully expanded. Compared to WT the hypocotyl of the *fri*$^1$ mutant is slightly elongated in B and R (Figs. 2, 3).

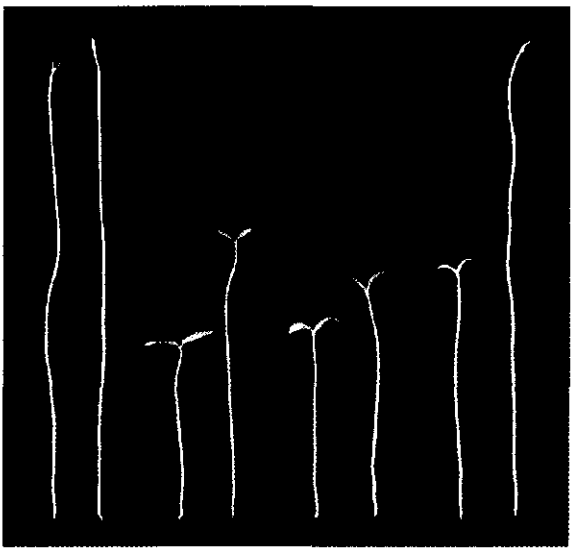


**Figure 2.** Phenotype exhibited by tomato seedlings grown for 7 days after emergence in darkness (D) and continuous broad band blue (B), red (R) and far-red (FR) light of 3 μmol $m^{-2}$ $s^{-1}$. For each treatment, the seedling on the left is the wild type (WT, cv. MM) and on the right is the *fri* mutant.

When grown in a 16 h WL/8 h D cycle for 28 days, the *fri* mutants are phenotypically barely distinguishable from WT, apart from their very slightly retarded growth (Fig. 4). This retarded growth becomes more obvious in plants grown in the greenhouse and the *fri* mutant plants wilt strongly on sunny days. Preliminary data indicate that detached leaves of *fri* mutant plants do not show an enhanced water loss, as is found in abscisic acid-deficient mutants where this is due to their inability to close their stomata (data not shown).

Mutants with a similar D phenotype in FR, but which exhibit hypocotyl inhibition by other wavelengths, have been shown to be phyA mutants in *Arabidopsis* (Nagatani et al. 1993; Parks and Quail 1993; Whitelam et al. 1993)

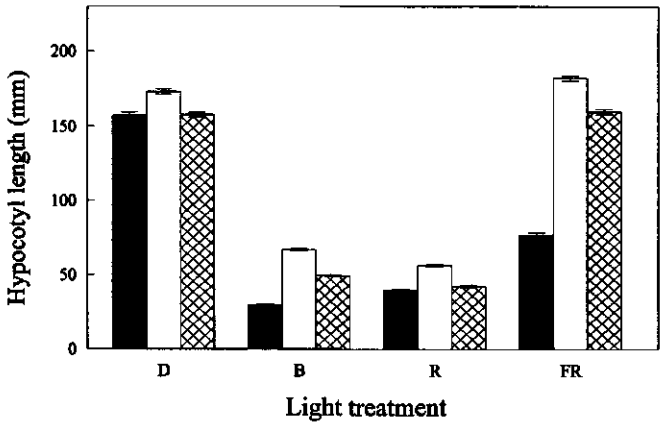


**Figure 3.** Hypocotyl length of tomato wild-type (WT, cv. MM, *filled bars*), *fri*$^{1}$ (*open bars*) and *fri*$^{2}$ (*hatched bars*) mutant seedlings after 7 days continuous darkness (D), blue (B), red (R) or far-red (FR) light of 3 μmol $m^{-2}$ $s^{-1}$. The mean hypocotyl length of at least 25 seedlings from each light treatment is plotted. Error bars represent the SE of the means.


**Figure 4.** Wild-type (WT) and *fri*$^{1}$ mutant tomato seedlings grown for 28 days in a 16 h white light (photosynthetically active radiation 100 μmol $m^{-2}$ $s^{-1}$)/ 8 h dark cycle at 25°C.

## Mapping

Recent research indicates the presence of at least five *phy* genes in tomato, at least three showing sequence homology to previously characterized *phy* genes in *Arabidopsis* (Hauser et al. 1994). None of these genes has yet been mapped to a specific chromosome in tomato. However, Sharrock et al. (1988), using an RFLP for a phytochrome-coding sequence from *Arabidopsis*, which they presumed was *phyA*, mapped this locus on chromosome 10. Since the phenotypic characteristics of the *fri* mutants, as shown in Figs. 2 and 3, resemble those of the phyA-deficient mutants of *Arabidopsis* (Nagatani et al. 1993; Parks and Quail 1993; Whitelam et al. 1993), linkage analysis was performed in $F_2$ populations derived from crosses of the *fri* mutants with a tester line homozygous recessive for several morphological markers on chromosome 10. Significant linkage was detected with all chromosome 10 markers used. Estimates of recombination percentages and map positions calculated from these data are given in Fig. 5.

If the *fri* mutants are shown to be phyA mutants, like those in *Arabidopsis*, then the *PHYA* gene of tomato is located on chromosome 10, a prediction confirmed by recent experiments (A. van Tuinen, unpublished data).

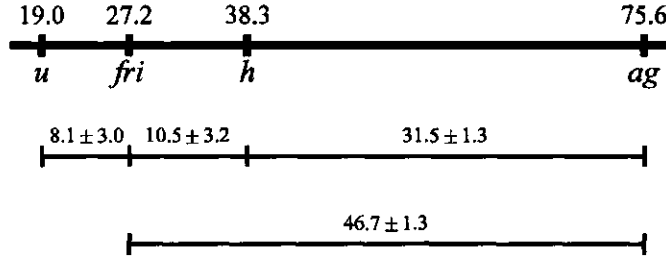


**Figure 5.** Location of the *fri* locus on tomato chromosome 10 and estimates of recombination percentages between the *fri* locus and morphological markers specific for chromosome 10. The map position of *u* is that on the linkage map published by Tanksley (1993).

## Immunochemical and *in vivo* spectrophotometrical analysis of phytochrome

In extracts of etiolated seedlings of both *fri* mutants, immunochemically detectable phyA polypeptide (PHYA) is essentially absent, yet both contain WT levels of a PHYB-like apoprotein. Results for the *fri*$^{1}$ mutant are given in Fig. 6, but similar results were obtained for *fri*$^{2}$ (data not shown). The very faint staining detectable in the *fri* mutants (estimated as less than 1% of WT band by dilution studies) is quite likely due to the fact that the antibody used for detection of PHYA, which was raised against pea PHYA, recognizes other minor phytochrome species. Immunoblot analysis with specific antibodies raised against tomato PHYA when available will prove whether the *fri* mutants are slightly leaky or not.

Spectrophotometric analysis showed that total phytochrome in the WT decreases during a 4 h R irradiation to the low level present in the *fri* mutants (Fig. 6). However, the low level of total phytochrome in the *fri* mutants is not susceptible to destruction during this 4 h irradiation with R, indicating that it is light-stable.

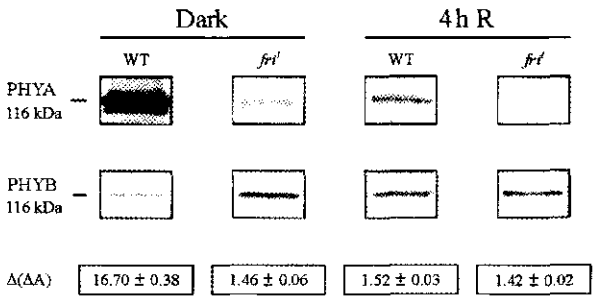


**Figure 6.** Immunoblot detection of phytochrome A and B polypeptide (PHYA and PHYB, respectively) and *in vivo* measurement of spectral activity of phytochrome in wild-type (WT, cv. MM) and *fri*$^{1}$ mutant seedlings. Dark-grown seedlings of 4-day-old or seedlings of the same age exposed to 4 h of red light (R) were used for the detection of PHYA and PHYB with monoclonal antibodies mAP5 and mAT1, respectively, in crude extracts. The phytochrome content was measured using a dual-wavelength spectrophotometer and is expressed as Δ (ΔA)/40 seedlings.

## Physiological characterization

*De-etiolation*

To obtain more detailed information about the resemblance between FR- and D-grown *fri* mutant seedlings, leaf area per cotyledon and chlorophyll content (characteristics of the de-etiolation process) were measured at different times following transfer of D and FR pretreated seedlings to WL. Figure 7 shows that the de-etiolation of the *fri* mutants follows the same kinetics, regardless of the pretreatment given. Cotyledons of the *fri* mutants expand in WL to a greater extent than those of the WT and have a higher chlorophyll content expressed on a per g fresh weight basis. In contrast to the *fri* mutants, the WT is unable to green after a pretreatment with FR and the fully expanded cotyledons lose their ability to produce chlorophyll, shrivel up and eventually the seedlings die. The *fri* mutants are blind to FR for the cotyledon expansion, but exhibit efficient chlorophyll biosynthesis and cotyledon expansion upon transfer to WL.

*Low fluence response for hypocotyl growth*

Phytochrome not only exists in multiple types, but also works via different modes: the low fluence response (LFR), which is R/FR reversible and a high irradiance response (HIR) which is irradiance- and duration-dependent (Mancinelli 1994).

In continuous low fluence broad-band R, the hypocotyl growth of the *fri* mutants is only slightly less inhibited than the WT (Figs. 2, 3). Spectrophotometric analysis showed that the PHYA pool is depleted after 4 h R (Fig. 6). This suggests the involvement of phyB and/or other stable pool type phytochrome(s) in the LFR of hypocotyl growth inhibition. It is therefore expected that the phyA-deficient *fri* mutants display normal R/FR reversibility for hypocotyl growth inhibition. To test this hypothesis the effect of pulses of R or R immediately followed by FR (both saturating for phytochrome photoconversion) given every 4 h on hypocotyl growth inhibition were investigated (Fig. 8). As expected the R/FR reversibility of the hy-

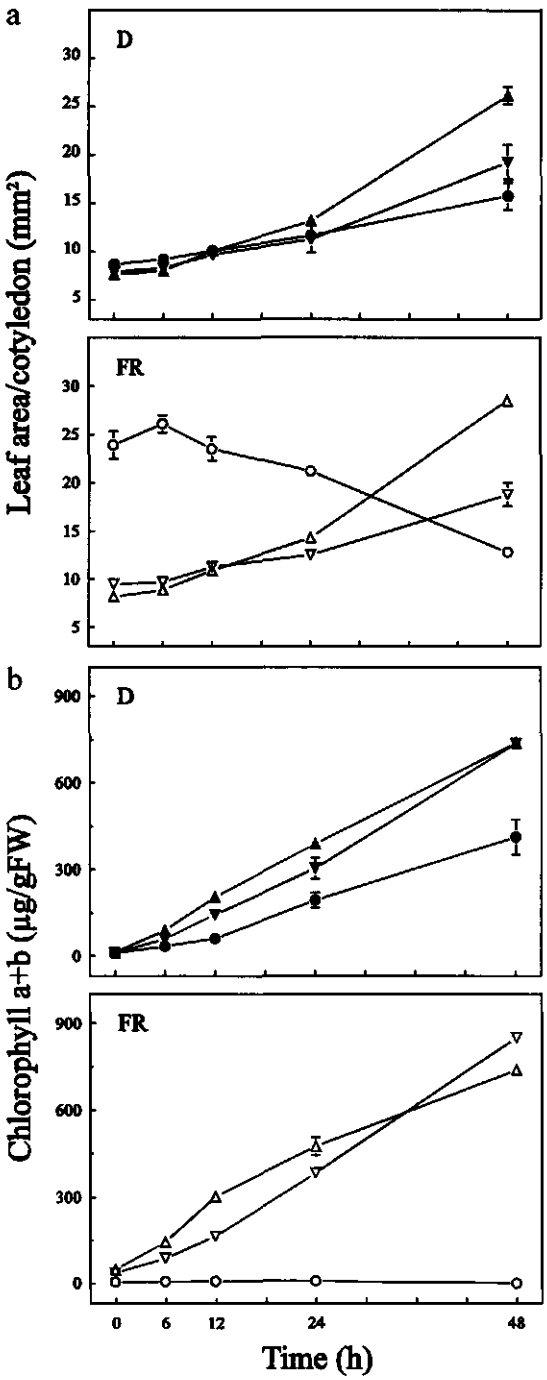


**Figure 7.** Leaf area (**a**) and chlorophyll content (**b**) of cotyledons of wild-type (WT, cv. MM, *circles*) and the *fri*$^{1}$ (*inverted triangles*) and *fri*$^{2}$ (*triangles*) mutant seedlings measured 0, 6, 12, 24 and 48 h after transfer to continuous white light (photosynthetically active radiation 120 µmol m$^{-2}$ s$^{-1}$). The seedlings were pre-treated with a 7 day period of darkness (D, *closed symbols*) or continuous far-red light (FR, 3 µmol m$^{-2}$ s$^{-1}$, *open symbols*). Error bars represent the SE of the mean.

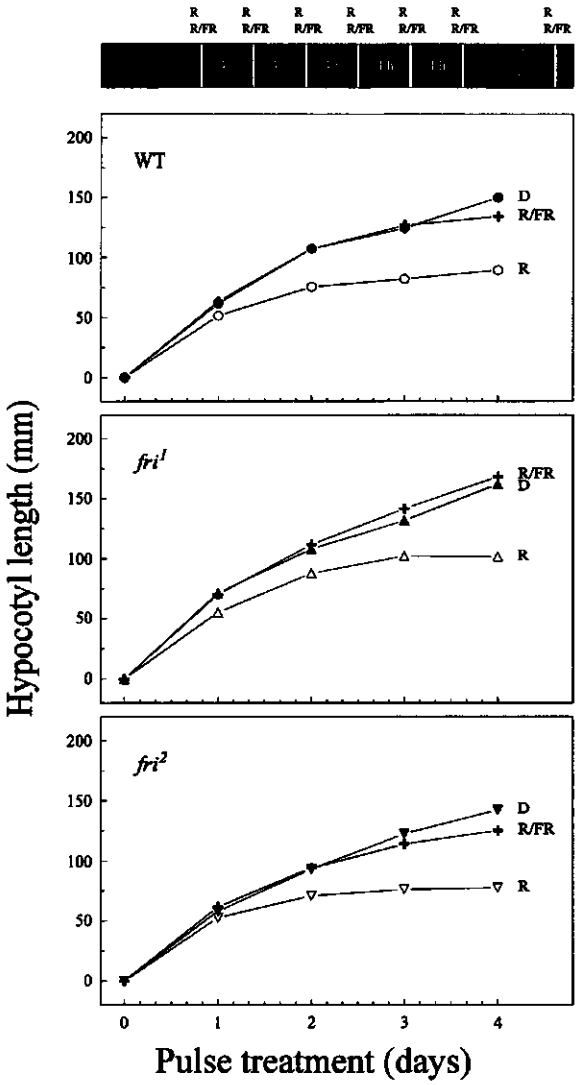


**Figure 8.** Hypocotyl length of wild-type (WT, cv. MM) and the *fri*[1] and *fri*[2] mutant seedlings treated with pulses of red light (R) or R immediately followed by far-red light (FR) and a dark (D) control. The R and FR pulses (both saturating for phytochrome photoconversion) were repeated every 4 h from the time of emergence. The SE in all cases was smaller than the symbols used.

pocotyl growth inhibition is retained in the *fri* mutants. The fact that the *au* mutant is more or less blind to R (Koornneef et al. 1985) and it is probably deficient in all types of phytochrome, gives an additional indication of the involvement of phyB and/or other phytochromes in the photocontrol of hypocotyl growth inhibition.

*End-of-day far-red light response*

The immunoblot analysis showed that D-grown seedlings, as well as WL-grown *fri* mutant plants have WT levels of a PHYB-like apoprotein. Because all mutants characterized as phyB deficient, i.e. *Arabidopsis hy3* (Reed 1993), cucumber *lh* (López-Juez et al. 1992) and *Brassica ein* (Devlin et al. 1992), lack the EODFR response, it is commonly accepted that this response is mediated by phyB. The *fri* mutants respond to end-of-day FR (EODFR) treatment with an increase in plant height qualitatively similar to WT (Fig. 9). This suggests that phyB functions normally and, moreover, that phyA has little or no influence on stem elongation in WL-grown tomato plants.

*Germination experiments*

Unlike the *au* mutant (Koornneef el al. 1985), seeds of the *fri*$^{1}$ mutant which germinate in darkness are inhibited by continuous FR (Fig. 10). We conclude that the phytochrome involved in this inhibition is not phyA.

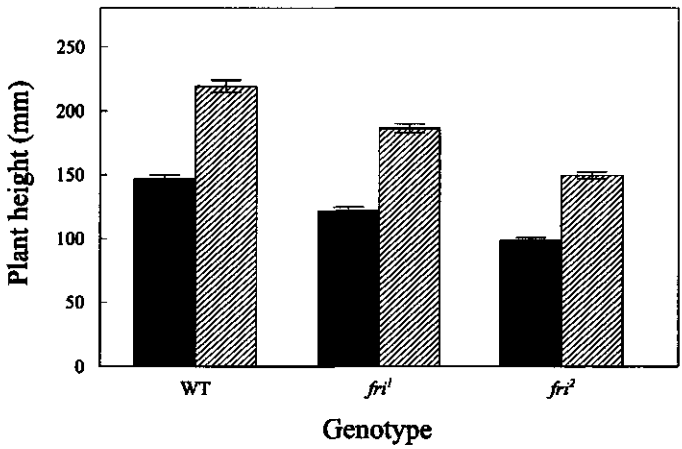


**Figure 9.** Plant height of 28-day-old wild-type (WT, cv. MM) and *fri*$^{1}$ and *fri*$^{2}$ mutant plants. After the 16 h daily white period (photosynthetically active radiation 60 µmol $m^{-2}$ $s^{-1}$), plants were either submitted to an immediate 8 h dark (D) period (*filled bars*) or given a 20 min FR pulse before the D period (*hatched bars*). Plant height was measured after 12 daily cycles with this end-of-day far-red light treatment. Error bars represent the SE of the mean.

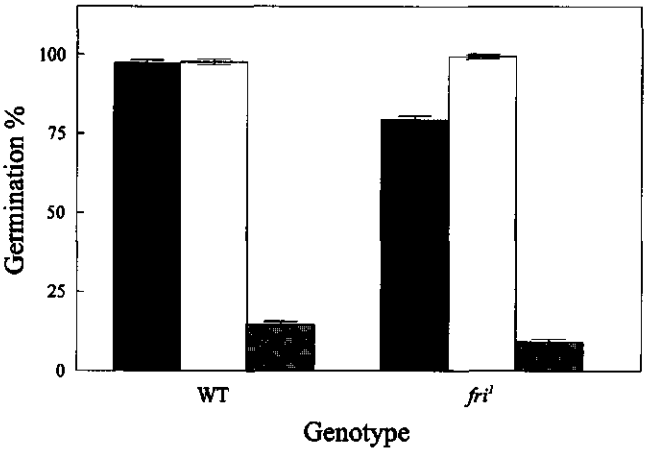


**Figure 10.** Final germination percentage of wild-type (WT, cv. MM) and *fri*$^{l}$ mutant seeds in darkness (*filled bars*), continuous red light (*open bars*) or far-red light (*shaded bars*). Error bars represent the SE of the mean.

## The role of phytochrome A in tomato

Phytochrome A-deficient mutants of tomato resemble the phyA mutants of *Arabidopsis*. In both species the absence of a FR-HIR for hypocotyl inhibition and the rather normal phenotype in WL are the most obvious characteristics. The effectiveness of phyA in the WT in continuous FR is explained by the lability of the FR-absorbing form of phyA (PfrA), the steady-state level of which is lower in FR, resulting in a higher integrated level of Pfr being maintained than in R (Mancinelli 1994).

In the *fri* mutant, FR is still able to photoconvert phytochrome (deplete Pfr), as shown in the EODFR, LFR and germination experiments in which the *fri* mutants resemble WT. These responses are therefore apparently mediated by other phytochromes, whose effectiveness is determined by the photoequilibrium between the R-absorbing form of phytochrome (Pr) and Pfr at any particular wavelength, meaning that R is more effective than FR. In

contrast to the *Arabidopsis* phyA mutants, the tomato *fri* mutants show a slightly reduced sensitivity to B and R, which allowed their initial selection as mutants at those wavelengths. The explanation for the R effect is that enough PfrA is presumably present during the prolonged exposure to contribute to the inhibition by R. The B effect can be explained because in tomato the effect of B is mediated via the phytochrome system (Mohr 1994), whereas in *Arabidopsis* B acts independently (Koornneef et al. 1980; Young et al. 1992). The most striking conclusion we can draw from the phenotypes of the *Arabidopsis* phyA mutants and the putative tomato phyA mutants reported here is the apparent absence of a role of this phytochrome species in light-grown plants (Fig. 4). While young *fri* mutant plants grown in the phytotron are only slightly retarded compared to the WT, the older plants in the greenhouse exhibit strong wilting on sunny days, which presumably accounts for their slower growth. The normal green leaf colour and growth habit of the *fri* mutants in general is in strong contrast to the phenotype of the *au* and yellow-green-2 (*yg-2*) mutants of tomato, which at both the seedling and adult plant stage are characterized by an elongated and pale green phenotype. We now assume that these effects, formerly attributed exclusively to the phyA deficiency of these mutants (Adamse et al. 1988), might be due to the deficiency of other phytochrome types and/or other effects of a defect in tetrapyrrole biosynthesis.

## Materials and methods

### Plant material

Mutants were obtained by treating seeds of tomato (*Lycopersicon esculentum* Mill.) cv. Moneymaker (MM) with ethyl methanesulphonate (EMS) for 24 h in darkness at 25°C (Koornneef et al. 1990). The population of $M_1$ plants was divided into groups of approximately ten plants and, from each group, $M_2$ seeds were harvested. The $M_2$ seed groups were screened for mutants with phenotypes deviating from WT in broad band R and B.

## Genetic characterization

Seedlings used in all types of genetic analyses were grown for 7 days after emergence under continuous FR (3 μmol $m^{-2}$ $s^{-1}$). Segregation ratios were determined by counting the number of seedlings with WT and mutant hypocotyl length. The mutants obtained were tested for allelism versus non allelism on the basis of non-complementation versus complementation to the WT phenotype in the $F_1$ plants.

Linkage analysis was done in $F_2$ populations derived from the cross of the *fri*$^2$ mutant with linkage marker stock LA780, obtained from C.M. Rick and homozygous recessive for markers *yv*, *c*, *h*, *ag*, *r* and *j*, but WT for the *u* gene (*U*$^+$) for which the cv. MM is recessive. Most of the *Fri*$^+$ seedlings did not survive the transition to WL after the 7-day FR period necessary for the identification of *fri* seedlings. The recombination percentages are based on the ratio of *fri* plants recessive for a given marker, as a fraction of the total number of *fri* seedlings tested (b) and estimated with the following formulae:

$$r_R = 100.\sqrt{b} \qquad r_C = 1 - r_R$$

$$s_r = \frac{100}{2\sqrt{b}} \cdot \sqrt{\frac{b(1-b)}{n}}$$

where

n = total number of plants; $r_R$= estimate of recombination percentage for repulsion phase, $r_C$ for coupling phase; $s_r$= standard deviation of r.

Map positions were calculated from the estimated recombination percentages using the computer program JOINMAP described by Stam (1993).

## Growth of plants for phytochrome assays

Seeds of the *fri*$^1$ mutant and WT were briefly incubated in WL with a 1% (v/v) solution of commercial bleach for 3 min and then washed thoroughly with running tap water. Seeds were sown on 0.6% (w/v) agar medium containing 0.46 g $l^{-1}$ of Murashige-Skoog salts (Gibco, Gaithersburg, MD) in plant tissue culture containers obtained from Flow Laboratories (McLean, VA). Seedlings were grown at 25°C for 4 days either in darkness or irradiated with R (20 μmol $m^{-2}$ $s^{-1}$; white fluorescent tubes; [FL20S.W.SDL.NU; National, Tokyo] filtered through 3 mm red acrylic [Shinkolite A102; Mitsibutsi Rayon, Tokyo]) for 4 h prior to harvest. The upper 1 cm of the hypocotyls, including the cotyledons, was harvested under dim green safelight after gently removing any remaining seedcoats. For *in vivo* spectrophotometry the samples were collected on ice and used immediately. The samples for immunoblotting were frozen in liquid nitrogen and stored at -80°C before analysis.

## *In vivo* phytochrome spectrophotometry

For the spectrophotometric measurements of phytochrome, about 0.4 g tissue (collected from 40 seedlings) were gently packed into a custom-built stainless steel cuvette with glass windows (10 mm in diameter and about 4 mm path length) and the phytochrome content was measured in a dual-wavelength spectrophotometer (model 557; Hitachi, Tokyo) using 730 and 800 nm measuring beams, which was equipped with an actinic irradiation unit for photoconverting the sample with saturating irradiations of R (30 s) and FR (60 s).

## Phytochrome extraction and immunoblotting

About 0.2 g (collected from 20 seedlings) frozen material was homogenized after adding 20 mg insoluble polyvinylpyrrolidone in 0.2 ml extraction buffer (100 mM Tris-HCl (pH 8.3), 50% v/v ethylene glycol, 140 mM ammonium sulphate, 56 mM 2-mercaptoethanol, 20 mM sodium bisulphate, 10 mM EDTA, 4 mM phenylmethylsulphonyl fluoride, 4 mM iodoacetamide), which was adjusted to 1 $\mu g\ ml^{-1}$ pepstatin A, 2 $\mu g\ ml^{-1}$ aprotinin and 2 $\mu g\ ml^{-1}$ leupeptin just before use in a microfuge tube at 4°C, using an homogenizer fitting the tube at full speed for 1 min. The homogenate was centrifuged at 0°C for 15 min at 18,000*g* in a microcentrifuge. The supernatant was mixed directly with 2 x SDS-sample buffer (Laemmli 1970) and dissolved at 100°C for 2 min. Then, 5 µl was immediately used for the SDS-PAGE and the remainder was stored at -20°C for further analysis.

Proteins were electrophoresed in 6.5% SDS-polyacrylamide gels, using prestained molecular mass standards (SDS-7B markers, Sigma, St. Louis, MO). The apparent molecular mass of these prestained markers was recalibrated using high molecular mass standards (SDS-6H markers, Sigma), and then electroblotted onto a nylon filter (FineBlott; Atto, Tokyo) in 100 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol. The membranes were blocked in a series of Tris-HCl buffer-saline Tween (TBST) solutions, all containing 20 mM Tris-HCl, pH 7.5, and varying Tween20 and NaCl concentrations: 2% (v/v) Tween and 500 mM NaCl for 3 min; 0.05% (v/v) Tween and 500 mM NaCl for 10 min; 0.05% (v/v) Tween and 150 mM NaCl. Incubation with the primary antibody was in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1% (w/v) fat-free milk powder. The monoclonal anti-PHYA and anti-PHYB antibodies used were mAP5 (Nagatani et al. 1985) and mAT1 (López-Juez et al. 1992) in dilutions of 2 $\mu g\ ml^{-1}$ and a 1:1 dilution of hybridoma culture supernatant, respectively. The incubation was at room temperature for 2 h, after washing three times with TBST, as at the end of the blocking; membranes were incubated with a 1:5000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Protoblot kit; Promega Corp., Madison, WI) for 45 min, washed, and stained for alkaline phosphatase according to the manufacturer's instructions.

## Pretreatment of the seeds

To obtain a higher germination percentage, the seeds used in the EODFR, pulse, and broad-band light experiments, and in the genetic analysis were pretreated before the final sowing. The seeds were sown in 9 x 9 cm refrigerator boxes on one layer of thick filter paper (T300-45mm, Schut BV, Heelsum, The Netherlands) moistened with 7.4 ml germination buffer (0.01 M $NaH_2PO_4.H_2O$, 0.01 M $K_2HPO_4.3H_2O$, 5 mM $KNO_3$, pH 7.5) and placed in wooden boxes. The boxes were put in a darkroom with a temperature of 25°C and relative humidity of 50-60%. After two days pretreatment, the seeds were planted out under dim green safe light in trays (30 x 20 x 5 cm) filled with a mixture of potting compost and sand (ratio 3:1) for the genetic analysis, pulse and broad-band light experiments.

## Continuous broad-band light experiment

Pretreated seeds sown in trays were incubated in D for 72 h at 25°C. The irradiation with continuous B, R and FR (3 μmol $m^{-2}$ $s^{-1}$) was started just before the seedlings emerged through the soil surface. The length of each hypocotyl was measured daily for 7 days with a ruler under dim green safe light. In addition, the hypocotyl length of plants grown in absolute D for the duration of the experiment was measured.

## De-etiolation experiments

The pretreated WT, *fri*$^1$ and *fri*$^2$ seeds were sown in trays and incubated in D for 56 h at 25°C. At that time the first seedlings started to emerge and the trays were either kept in D or transferred to FR (3 μmol $m^{-2}$ $s^{-1}$). Seedlings emerging during a following 12-h period were marked, allowed to grow for 7 days and then transferred to WL (photosynthetically active radiation [PAR, 400-700 nm], 110 μmol $m^{-2}$ $s^{-1}$). At 6, 12, 24 and 48 h after transfer to WL, cotyledon area and chlorophyll content of the cotyledons were measured as indicators of de-etiolation.

Cotyledon area was measured with a leaf analysis system of Skye Instruments Ltd (Powys, UK). Chlorophyll was extracted using a method adapted from that described by Hiscox and Israelstam (1979). Samples of ten cotyledons were weighed, placed in glass tubes and incubated in D for 24 h at 65°C with 5 ml DMSO and assayed immediately after cooling down to room temperature. Chlorophyll content was calculated using the equations for ethanol published by Lichtenthaler and Wellburn (1983).

## Pulse experiment

Pretreated seeds sown in trays were incubated in D for 48 h at 25°C. Pulses of R (3 min, 7.8 μmol $m^{-2}$ $s^{-1}$) or R immediately followed by FR (6 min, 6.7 μmol $m^{-2}$ $s^{-1}$), both saturating for attaining phytochrome photoequilibrium, were given every 4 h beginning at the time of emergence of the first seedlings. During the pulse irradiation every emerging seedling was marked, enabling the measurement of hypocotyl growth of each seedling after the appropriate number of pulses (6, 12, 18 or 24).

## End-of-day far-red light experiments

Pretreated seeds were sown in 7 x 7 x 8 cm plastic pots filled with a mixture of potting compost and sand (ratio 3:1) and grown for 12 days in a phytotron with a daily irradiation schedule of 16 h WL (PAR, 160 μmol $m^{-2}$ $s^{-1}$)/8 h D at 25°C and relative humidity of 65-70%. At day 13, the plants were transferred to cabinets and allowed to adjust to the lower level of WL (PAR, 60 μmol $m^{-2}$ $s^{-1}$) for 4 days. Plants were then selected for uniform height and after the daily WL period received an immediate 20 min FR irradiation (4.6 μmol $m^{-2}$ $s^{-1}$) before the D period. The controls were grown in an identical cabinet and received no FR irradiation. Plant height was measured after 12 days of EODFR treatment.

## Light sources

The broad-band B and R cabinets used in the initial screen for mutants, together with the FR cabinets used in the additional broad-band spectrum scan of the newly isolated mutants, were the same as those described by Koornneef et al. (1980).

The genetic analysis, broad-band, pulse, EODFR and germination experiments were carried out in new light cabinets at Wageningen. The lamp compartment provides space for five fluorescent tube fittings with reflectors which are controlled by a continuously variable dimmer unit and time clocks (Nijssen Light Division BV, Wageningen, The Netherlands). These cabinets were used for irradiation with WL, B, R, and FR using the tubes and filters described below. The fluence rates and exposure times used are given in the description of each experiment.

WL was obtained from Philips TLD36/84 fluorescent tubes; the B source was the same as described in Koornneef et al. (1980); R was obtained by filtering the light from Philips TL40/103339 fluorescent tubes through two layers of primary red filter (Lee, Flashlight Sales BV, Utrecht, The Netherlands); FR continuous broad-band: Sylvania F48T12/232/HO with one layer of primary red and one layer of dark green filter (Lee). For pulse and EODFR experiments, Sylvania F48T12/232/VHO tubes were wrapped with one layer of dark green and one layer primary red filter (Lee). All light measurements were made using a LI1800/12 spectroradiometer (Li-cor, Lincoln, NE).

**Acknowledgements** This research was supported by grants to A.v.T. and L.H.J.K. from the Foundation for Life Sciences (SLW) formerly the Foundation for Biological Research (BION) which is subsidized by the Netherlands Organization for Scientific Research (NWO). We thank Corrie Hanhart and Mariëlle Schreuder for technical assistance, Willem Tonk for assistance in the design and construction of the broad-band light sources at Wageningen, Martijn van Eeden for the data of the germination experiment, Enrique López-Juez for assistance with Western blotting and Dr. S. Yoshida for use of greenhouse facilities at Riken.

# Chapter 3

# A temporarily red light-insensitive, mutant of tomato lacks a light-stable, B-like phytochrome

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Published in *Plant Physiol* (1995) **108:** 939-947

**Abstract** *We have selected four recessive mutants in tomato (*Lycopersicon esculentum *Mill.) that, under continuous red light (R), have long hypocotyls and small cotyledons compared to wild type (WT), a phenotype typical of phytochrome B (phyB) mutants of other species. These mutants, which are allelic, are only insensitive to R during the first 2 days upon transition from darkness to R, and therefore we propose the gene symbol* tri *(temporarily red light insensitive). White light-grown mutant plants have a more elongated growth habit than that of the WT. An immunochemically and spectrophotometrically detectable phyB-like polypeptide detecteble in the WT is absent or below detection limits in the* $tri^1$ *mutant. In contrast to the absence of an elongation growth response to far-red light (FR) given at the end of the daily photoperiod (EODFR) in all phyB-deficient mutants so far characterized, the* $tri^1$ *mutant responds to EODFR treatment. The* $tri^1$ *mutant also shows a strong response to supplementary daytime FR. We propose that the phyB-like phytochrome deficient in the tri mutants plays a major role during de-etiolation and that other light-stable phytochromes can regulate the EODFR and shade avoidance responses in tomato.*

# Introduction

The red light (R)/far-red (FR)-absorbing phytochrome photoreceptor system plays a leading role in the regulation of development throughout the life cycle of plants. Examples of the light-mediated processes that it influences are seed germination, de-etiolation (inhibition of hypocotyl growth, opening of the apical hook, expansion of the cotyledons, development of chloroplasts, accumulation of anthocyanin), shade avoidance, and induction of flowering (Kendrick and Kronenberg, 1994).

In *Arabidopsis thaliana* the phytochrome family consists of at least five different genes referred to as *PHYA* through *PHYE*, which encode apophytochrome PHYA through PHYE, and form holophytochrome phyA

through phyE after insertion of the chromophore, respectively (Quail, 1994). Recent research (Hauser et al. 1994; Pratt 1995) reports the presence of an even more complex gene family in tomato (*Lycopersicon esculentum* Mill.). Mutants deficient in one specific type of phytochrome are needed if we are to unravel the roles of the different phytochrome species in photomorphogenesis. So far, three types of phytochrome mutants have been characterized: (a) Mutants that are thought to be deficient in all types of phytochrome, are probably caused by a defect in the biosynthesis of the common phytochrome chromophore, like the *hy1* and *hy2* mutants of *Arabidopsis* (Parks and Quail 1991) and the *pew* mutants of *Nicotiana plumbaginifolia* (Kraepiel et al. 1994). In tomato the *aurea* (*au*) and *yellow green-2* (*yg-2*) mutants (Koornneef et al. 1985) are possible candidates for tomato chromophore mutants (Sharma et al. 1993; Van Tuinen et al. 1995a). (b) PhyA-deficient mutants have been reported in *Arabidopsis* (Dehesh et al. 1993; Nagatani et al. 1993; Parks and Quail 1993; Whitelam et al. 1993) and more recently in tomato (Van Tuinen et al. 1995a). (c) Mutants that lack a light-stable, phyB-like protein have been reported in several species, including cucumber (López-Juez et al. 1992), *Brassica rapa* (Devlin et al. 1992) and *Arabidopsis*. Only in the case of the *Arabidopsis hy3* (= *phyB*) mutant has it been proven that the mutation is located in the *PHYB* gene itself (Reed et al. 1993). The phyB-deficient mutants are characterized by their failure to de-etiolate in continuous R, resulting in a long hypocotyl and small cotyledons, the absence of an end-of-day FR (EODFR) response, an elongated stature, and a slightly reduced chlorophyll content when grown in white light (WL).

In view of the large number of phytochrome genes in tomato, there is a need for more type-specific phytochrome mutants to enable the physiological roles of the different phytochromes to be elucidated. The fact that phyB-deficient mutants already described in other species have a common phenotype has enabled us to screen for phyB-deficient mutants in tomato under WL and continuous R. This paper presents the isolation and characterization of such mutants in tomato.

# Results and discussion

## Mutant isolation and genetic characterization

The $M_2$ populations of tomato derived from ethyl methanesulphonate-treated seeds were screened under WL (GT background) or continuous blue light (B) and R (MM background). Two independently induced mutants C66 and B10, were selected for their slightly longer hypocotyls in WL in the $M_2$ generation derived from 1650 $M_1$ plants (experiments II & III in Koornneef et al. 1990). A third mutant, sc72, was isolated because of its longer hypocotyl in WL as a somaclonal variant in experiments described by Van den Bulk et al. (1990), which involved testing of 1052 progenies of regenerated MM plants. The fourth mutant, 2-19ARL, was selected for its longer hypocotyl under continuous broad-band R in $M_2$ material that was described by Van Tuinen et al. (1995a). In broad-band spectral study experiments, all the mutants showed a reduced hypocotyl growth inhibition in R. Genetic complementation analysis showed that the four mutants were allelic. Since the mutants are insensitive to R only during the first 2 days of R treatment (see Fig. 7), we propose the gene symbol *tri* (temporarily red light insensitive) for these mutants. The different alleles have been numbered in order of isolation, i.e. $tri^1$ = C66; $tri^2$ = B10; $tri^3$ = sc72; $tri^4$ = 2-19ARL. Under continuous R the progeny of selfed $F_1$ plants from the cross between the new mutant lines and the WT parent segregated in a 3:1 ($\chi^2 = 0.92$, $P > 0.05$ for the $F_2$ WT x $tri^1$ [Fig. 1]) ratio of normal to elongated hypocotyls and normal to small cotyledons expected for a monogenic recessive mutation. Figure 1 shows that the hypocotyl length of the heterozygote $F_1$ is slightly longer than that of the WT parent. This partial dominance of the mutation is a feature expected for a rate-limiting component such as a photoreceptor, and has previously been observed for the phyB-deficient *hy3* (Koornneef et al. 1980) and the phyA-deficient *fhy2* (= *phyA*) (Whitelam et al. 1993) mutants of *Arabidopsis*, the *fri* mutants of tomato (Van Tuinen et al. 1995a), and the $ma_3^R$ mutant of

*Sorghum bicolor*, which lacks a phytochrome that predominates in green tissue (Childs et al. 1992; Foster et al. 1994).

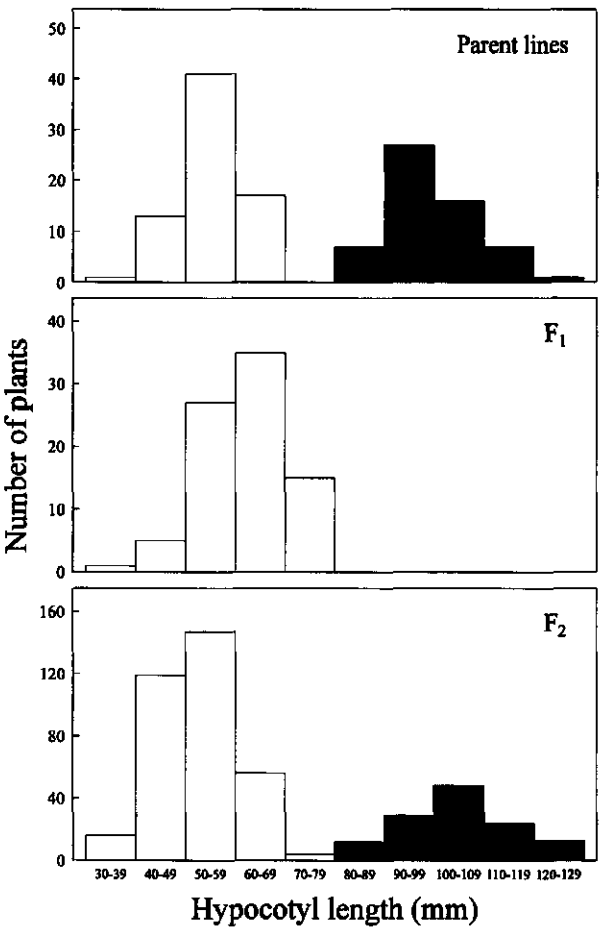


**Figure 1.** Frequency distribution of hypocotyl length of tomato seedlings of the wild type (*open bars*), *tri*$^{1}$ mutant (*filled bars*), $F_1$ and $F_2$ generations after 7 days of continuous red light (3 μmol $m^{-2}$ $s^{-1}$).

## Phenotypes of the *tri* mutants

Under broad-band spectral light sources we have examined hypocotyl length, cotyledon area, and anthocyanin and chlorophyll content for plants homozygous for the *tri* mutation. There is no difference between the WT and $tri^1$ and $tri^3$ mutants in D, and under FR. In B the hypocotyl of the $tri^1$ and $tri^3$ mutants are slightly elongated compared to their respective WT. In R, however, the mutants are characterized by a longer hypocotyl, less anthocyanin, and smaller, darker-green cotyledons than the WT (Figs. 2 and 3). There is an inverse relationship between cotyledon area and chlorophyll content expressed on a fresh weight basis, suggesting that total chlorophyll production is little influenced by the mutation. Figure 3 also shows that anthocyanin accumulation and cotyledon area are both affected by genetic background. However, the $tri^1$ and $tri^3$ mutant phenotypes are qualitatively similar.

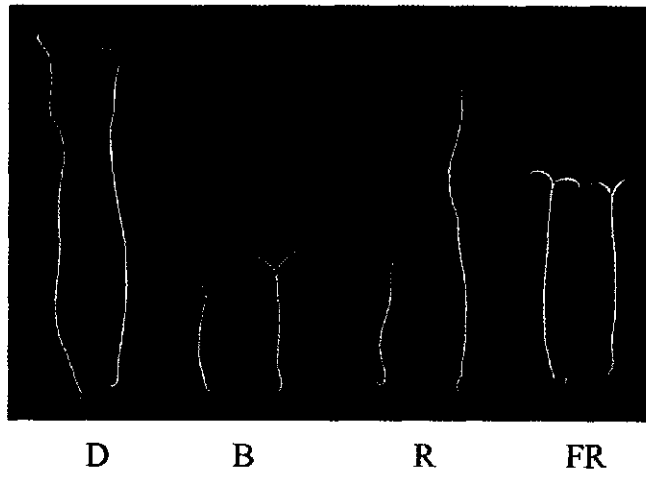


**Figure 2.** The phenotype of tomato seedlings grown for 7 days after emergence in darkness (D) and continuous broad band blue (B), red (R) and far-red (FR) light of 3 μmol $m^{-2}$ $s^{-1}$. For each treatment the seedling on the left is the wild type and that on the right is the $tri^1$ mutant.

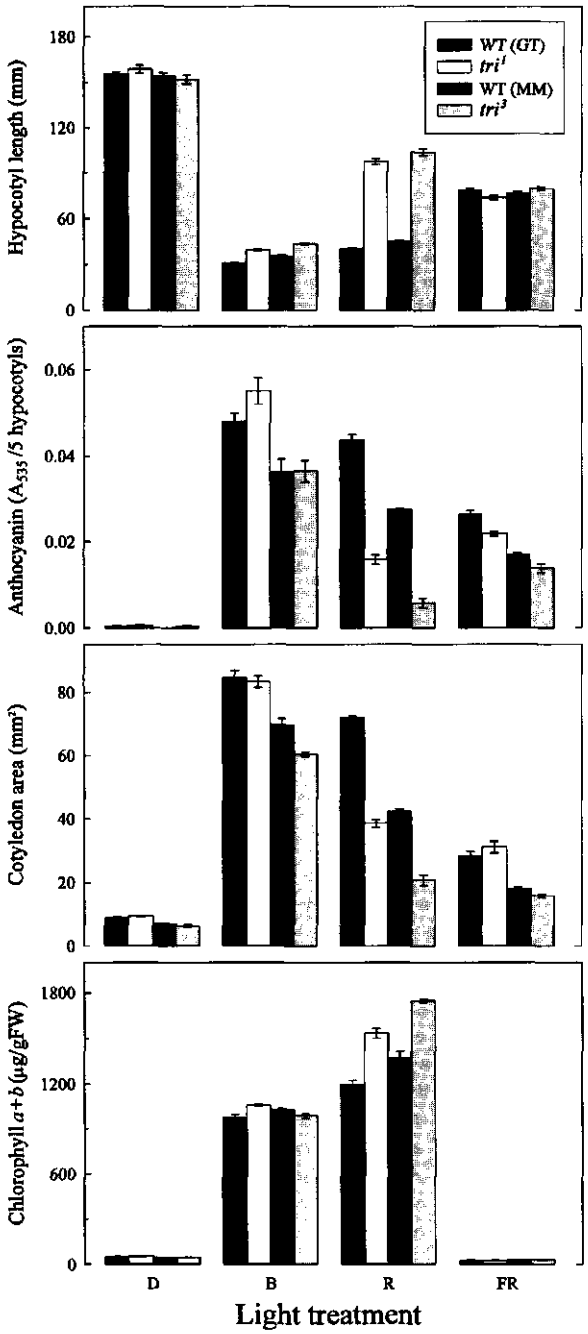


**Figure 3.** Hypocotyl length, anthocyanin content, cotyledon area and chlorophyll content of tomato wild-type (WT), *tri*$^{1}$ and *tri*$^{3}$ mutant seedlings after 7 days continuous darkness (D), blue (B), red (R) or far-red (FR) light of 3 µmol $m^{-2}$ $s^{-1}$. The mean hypocotyl length of 20 seedlings from each light treatment is plotted. Error bars represent the SE of the means.

When grown in a 16 h WL/8 h D cycle the hypocotyl of the *tri*$^{1}$ mutant is slightly elongated at the seedling stage (Fig. 4). The difference in height between the *tri* mutants and their isogenic WTs becomes more apparent with age, and the young immature leaves possess less anthocyanin (data not shown).

Mutants with a similar phenotype in R, but which exhibit hypocotyl inhibition by other wavelengths, have been shown to be phyB deficient in *Arabidopsis* (Nagatani et al. 1991; Reed et al. 1993) or to lack a phyB-like phytochrome, as in the cucumber *lh* mutant (López-Juez et al. 1992) and the *Brassica ein* mutant (Devlin et al. 1992).

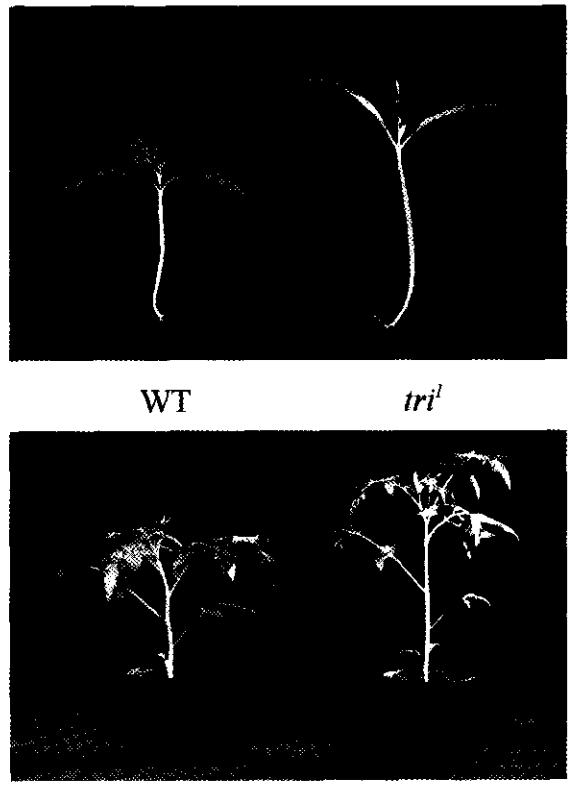


**Figure 4.** Wild-type (WT) and *tri*$^{1}$ mutant tomato seedlings grown for 7 (top) or 28 (bottom) days in a 16 h white light (photosynthetically active radiation 175 and 100 µmol $m^{-2}$ $s^{-1}$, respectively)/8 h dark cycle at 25°C.

## Immunochemical and *in vivo* spectrophotometrical analysis of phytochrome

In extracts of etiolated seedlings of the *tri*$^{1}$ mutant, an immunochemically detectable phyB-like polypeptide (PHYB), readily detectable in WT extracts, is absent or below detection limits, yet it contains WT levels of phyA apoprotein (Fig. 5). Spectrophotometric analysis showed that the light-labile phyA pool is depleted after 4 h R (Van Tuinen et al. 1995a). The difference in Δ(ΔA) between the WT and the reduced level observed in the *tri*$^{1}$ mutant presumably represents the lack of a phyB-like, stable apoprotein in the *tri*$^{1}$ mutant. These results resemble those found by Peters et al. (1991) for the cucumber *lh* mutant, which also lacks a phyB-like apoprotein (López-Juez et al. 1992).

Immunoblot analysis of WL-grown tissue (Fig. 6) revealed that the phyB-like apoprotein is not only absent in etiolated mutant seedlings, but remains absent in 3-week-old plants.

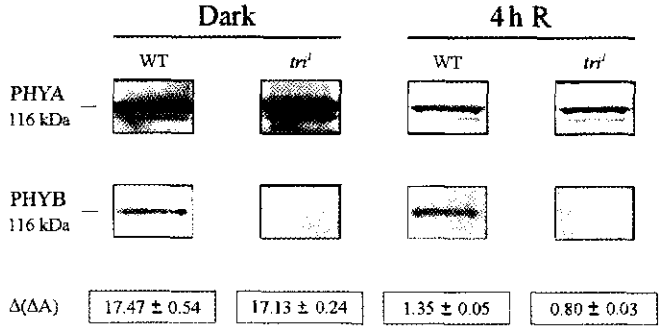


**Figure 5.** Immunoblot detection of phytochrome A and B polypeptide (PHYA and PHYB, respectively) and *in vivo* measurement of spectral activity of phytochrome in wild-type (WT) and *tri*$^{1}$ mutant seedlings. Dark-grown 4-day-old seedlings or seedlings of the same age exposed to 4 h of red light (R) were used for the detection of PHYA and PHYB with monoclonal antibodies mAP5 and mAT1, respectively, in crude extracts. The phytochrome content was measured using a dual-wavelength spectrophotometer and is expressed as Δ(ΔA)/40 hypocotyl sections.

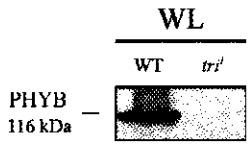


**Figure 6.** Immunoblot detection of phytochrome B polypeptide (PHYB) with monoclonal antibody mAT1 in crude extracts of 21 day old wild-type (WT) and *tri*$^{1}$ mutant plants. Plants were grown in a phytotron at 25°C with a daily regime of 16 h white light (photosynthetically active radiation 150 μmol $m^{-2}$ $s^{-1}$)/8 h of dark.

## Low fluence response experiments

Phytochrome not only exists in multiple types, but also works via different modes: the low fluence response (LFR), which is R/FR reversible, and a high irradiance response (HIR), which is irradiance and duration dependent (Mancinelli 1994).

The phyB-like-deficient *tri* mutants, in contrast to the phyA-deficient *fri* mutants, show little hypocotyl growth inhibition in continuous broad-band R (Fig. 3). Since spectrophotometric analysis has shown that the phyA pool is depleted after 4 h R (Van Tuinen et al. 1995a), phyB and/or other light-stable type phytochrome(s) must play the major role in growth inhibition under continuous R. We tested the involvement of a LFR in hypocotyl growth inhibition with pulses of R or R immediately followed by FR (both saturating for phytochrome photoconversion) given every 4 h on hypocotyl growth inhibition. Figure 7 shows that the *tri*$^{1}$ mutant is insensitive to R only during the first 2 days of pulse treatment. Thereafter the inhibitory effect of R on hypocotyl elongation growth and FR reversibility are retained in the *tri* mutants. Since the phyB-like phytochrome is still below detection limits in older WL-grown plants of the *tri*$^{1}$ mutant (Fig. 6), the temporal appearance of responsiveness to R cannot be explained by a delay in appearance of the phyB-like phytochrome.

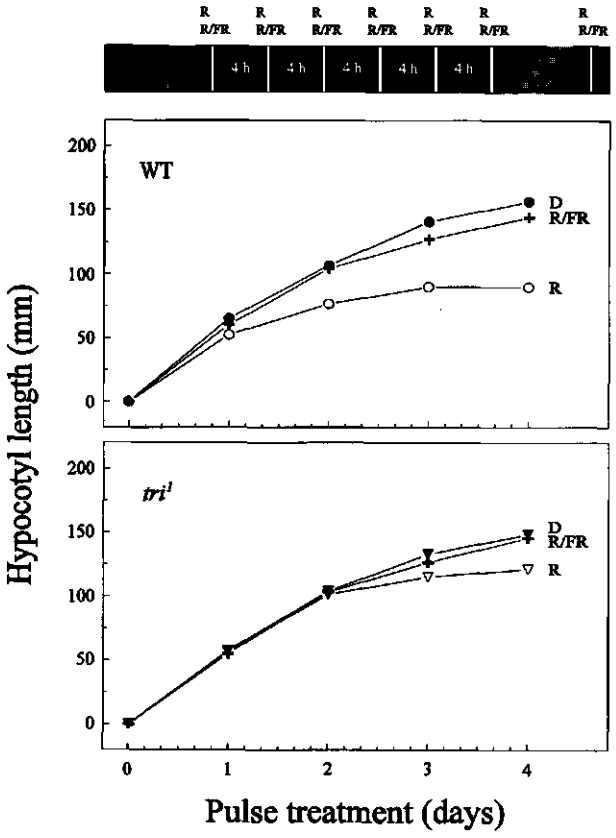


**Figure 7.** Hypocotyl length of wild-type (WT) and the *tri*$^{1}$ mutant seedlings treated with pulses of red light (R) or R immediately followed by far-red light (FR) and a dark (D) control. The R and FR pulses (both saturating for phytochrome photoconversion) were repeated every 4 h from the time of emergence. The SE in all cases was smaller than the symbols used.

## Delayed red light experiments

To test whether the 2 day period of insensitivity to R for hypocotyl growth inhibition of the *tri* mutant depends on a temporal pattern of development or the time after a transfer from D, seedlings were grown in D or continuous R, or were grown and kept in D for 1, 2 (data not shown), or 3 day(s) after emergence before transfer to continuous R. The WT exhibits a significant response to R within 24 h after transfer from D to R, whereas the *tri*$^{1}$ mutant stays insensitive to R for 2 days following the transfer from D to R (Fig. 8) irrespective of the length of the preceding D period.

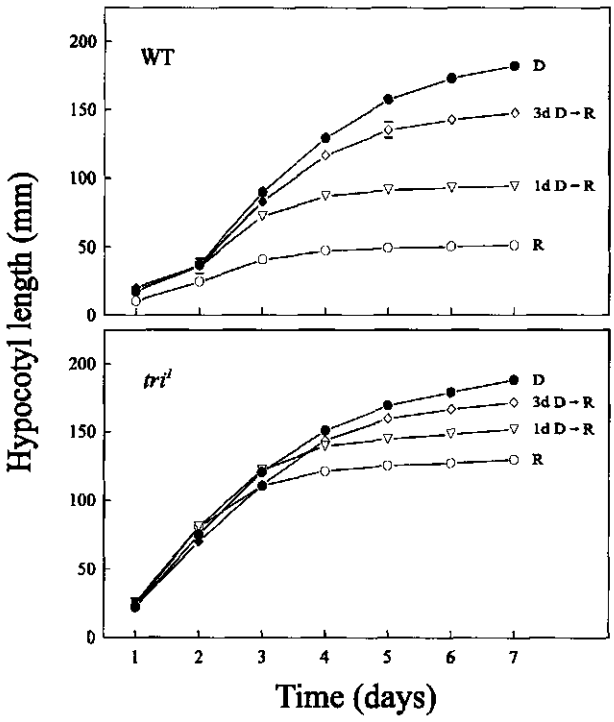


**Figure 8.** Hypocotyl length of wild-type (WT) and the *tri*$^{1}$-mutant seedlings. Seedlings were grown for 7 days in darkness (D, ●), continuous red light (R, 3 µmol $m^{-2}$ $s^{-1}$, ○) or transferred to R after a D period of 1 day (1d, ▽) or 3 days (3d, ◇).

## Responses to end-of-day and supplementary daytime far-red light

The *tri*$^{1}$ mutant responds to EODFR treatment with an increase in plant height that is quantitatively similar to WT, although the absolute height of the mutant is somewhat greater (Fig. 9).

Both WL-grown WT and *tri*$^{1}$ mutant plants show also a typical promotion of elongation growth in response to supplementary FR during the daily photoperiod (Fig. 10). The response is apparently slightly less in the *tri*$^{1}$ mutant, but this could be due to attainment of the maximal growth possible under these conditions.

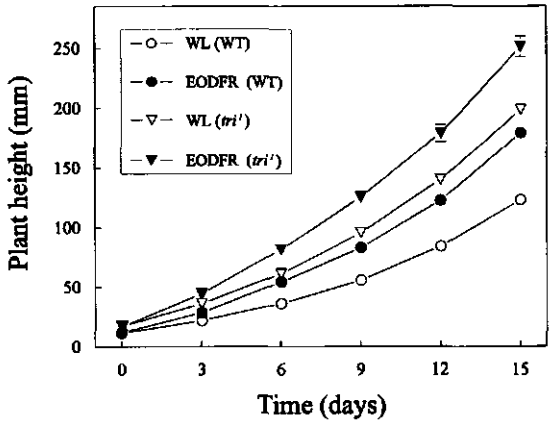


**Figure 9.** Plant height of wild-type (WT) and *tri*$^{1}$ mutant plants. After the 16 h daily white (WL, photosynthetically active radiation 125 µmol $m^{-2}$ $s^{-1}$) period, plants were either submitted to an immediate 8 h dark (D) period or given a 20 min far-red (FR) pulse before the D period. Plant height was measured every 3rd day during a 15 day period of daily cycles with an end-of-day far-red light (EODFR) treatment. Error bars represent the SE of the mean.

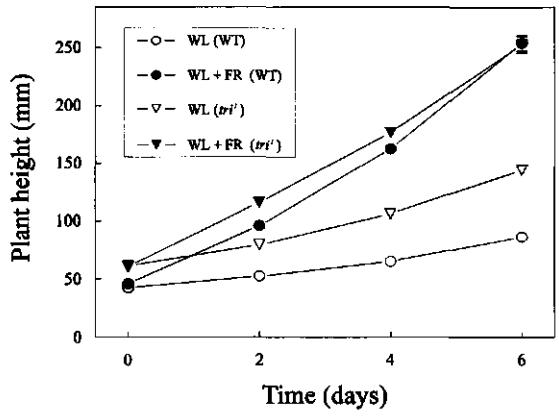


**Figure 10.** Plant height of wild-type (WT) and *tri*$^{1}$ mutant plants under conditions of 16 h white light (WL, photosynthetically active radiation 250 µmol $m^{-2}$ $s^{-1}$)/8 h dark or the same WL photoperiod with supplementary far-red light (FR; WL + FR). Error bars represent the SE of the mean.

## The role of phytochrome B in tomato

The *tri*$^{1}$ mutant of tomato resembles the *hy3* (= *phyB*) mutant of *Arabidopsis* (Reed et al. 1993), the cucumber *lh* mutant (Adamse et al. 1987; López-Juez et al. 1992) and the *Brassica ein* mutant (Devlin et al. 1992) in such characteristics as a longer hypocotyl, reduced anthocyanin content, and smaller cotyledons in continuous broad-band R (Fig. 3) and the absence of a phyB-like apoprotein compared to the WT (Fig. 5).

In contrast to the *hy3*, *ein* and *lh* mutants, in which the inhibition of hypocotyl growth and cotyledon expansion in R is essentially lost, the *tri*$^{1}$ mutant is only insensitive to R during the first 2 days upon transition from D to R (Figs. 7 and 8). This results in a phenotype in continuous R with longer hypocotyls and smaller cotyledons, but less extreme, for instance, than that of the almost completely R- and FR-blind tomato *au* mutant (Koornneef et al. 1985).

The EODFR response (Fig. 9) and the effect of supplementary daytime FR (Fig. 10), commonly accepted to be regulated by phyB (Adamse et al. 1987; Devlin et al. 1992; López-Juez et al. 1992; Reed et al. 1993), however, are present in the *tri*$^{1}$ mutant. Preliminary experiments indicate that another phyB-mediated response, simulated phototropism as a result of covering one of the cotyledons with aluminium foil, is also present in the *tri* mutant (results not shown). The fact that the *tri* mutant, which lacks a phyB-like apoprotein, is only temporary insensitive to R and still responds to EODFR and supplementary daytime FR treatment distinguishes this mutant from previously described phyB-deficient mutants in other species (Whitelam and Smith 1991). The temporal insensitivity to R can be explained by assuming that other stable phytochromes can perform physiological functions similar to the phyB-like phytochrome absent in the *tri*$^{1}$ mutant. The identification of multiple phytochrome genes, including two phyB-like genes, in tomato (Hauser et al. 1994; Pratt 1995) implies that this might be possible. However, the complete insensitivity of the *tri* mutants to R in the 2 days after the transition from D to R could suggest that at this stage the other phytochrome

genes are not expressed or do not function. We have eliminated the possibility that the appearance of sensitivity is due to a delayed appearance of the phyB-like phytochrome in the *tri*$^{1}$ mutant, since it is still below detection limits in light-grown plants.

The *tri* mutants are the first examples of mutants that indicate that a process thought to be regulated by phyB exclusively seems, at least in tomato, to involve more than one light-stable type of phytochrome. It is interesting to note that the PHYB antibody used in this study (mAT1) was the same as that which detected the absence of a phyB-like apoprotein in the cucumber *lh* mutant (López-Juez et al. 1992), again supporting the difference between tomato and other species. It should be noted, however, that the apparent insensitivity to supplementary FR on flowering of the *hy3* mutants is not found when they are studied in a more extreme genetic, and therefore a more sensitive, background (Halliday et al. 1994), which has also been explained by the proposal of the action of other light-stable phytochromes. A more detailed molecular analysis of the different phytochrome genes in the *tri* mutants is required for the ultimate proof of the relationship between the physiological defects of the *tri* mutants and a specific phytochrome gene. A detailed analysis of the various phytochrome-induced processes, using well-characterized mutants and double mutants lacking specific phytochrome types, will be essential for our full understanding of light-controlled plant development.

## Materials and methods

### Plant material

Mutants were obtained by treating seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar Moneymaker (MM) and breeding line GT with ethyl methanesulphonate for 24 h in D at 25°C (Koornneef et al. 1990). The $M_2$ seed groups were screened for mutants with phenotypes deviating from WT in WL and broad-band R and B. One mutant with an elongated phenotype in WL, due to somaclonal variation, was also isolated in the progeny of plants regenerated from tissue culture described by Van den Bulk et al. (1990).

## Genetic characterization

Seedlings used in all types of genetic analyses were grown for 7 days after emergence under continuous R (3 μmol $m^{-2}$ $s^{-1}$). Segregation ratios were determined by counting the number of seedlings with a WT or mutant hypocotyl length. The mutants obtained were tested for allelism versus non allelism on the basis of noncomplementation versus complementation to WT phenotype in the $F_1$ plants. The subsequent $F_2$ generation was retested in R and confirmed the $F_1$ data.

## Growth of plants for phytochrome assays

Seeds of the *tri*$^{1}$ mutant and WT were briefly surface sterilized under WL with a 1% (v/v) solution of commercial bleach for 3 min and then washed thoroughly with running tap water. Seeds were then sown on 0.6% (w/v) agar medium containing 0.46 g $l^{-1}$ of Murashige-Skoog salts (Gibco. Gaithersburg, MD) in plant tissue culture containers obtained from Flow Laboratories (McLean, VA). Seedlings were grown at 25°C for 4 days either in D or irradiated with R (20 μmol $m^{-2}$ $s^{-1}$; white fluorescent tubes [FL20S.W.SDL.NU; National, Tokyo] filtered through 3-mm red acrylic [Shinkolite A102; Mitsibutsi Rayon, Tokyo]) for 4 h prior to harvest. The upper 1 cm of the hypocotyls, including the cotyledons, were harvested under a dim-green safelight after gently removing any remaining seedcoats. For *in vivo* spectrophotometry the samples were collected on ice and used immediately. The samples for immunoblotting were frozen in liquid nitrogen and stored at -80°C before analysis.

Plants were also grown in pots containing a 2:3 (v/v) granular clay-based compost:vermiculite mixture in a phytotron (Koitotron KG-206HL-D, Koito, Tokyo) at 25°C with a daily regime of 16 h white fluorescent light (photosynthetically active radiation [PAR, 400-700 nm], 150 μmol $m^{-2}$ $s^{-1}$)/8 h of D. Twenty-one days after sowing, leaf samples were harvested (second and third leaves, only leaflets were used), frozen in liquid nitrogen, and stored at -80°C before extraction for immunoblotting.

## *In vivo* phytochrome spectrophotometry

For the spectrophotometric measurements of phytochrome, about 0.4 g tissue (collected from 40 seedlings) was gently packed into a custom-built stainless-steel cuvette with glass windows (10 mm in diameter and about a 4-mm pathlength), and the phytochrome content was measured as the difference in absorbance difference between 730 and 800 nm [Δ(ΔA)] in a dual-wavelength spectrophotometer (model 557; Hitachi, Tokyo), which was equipped with an actinic irradiation unit for photoconverting the sample with saturating irradiations of R (30 s) and FR (60 s).

## Phytochrome extraction and immunoblotting

About 0.2 g (collected from 20 seedlings) of frozen material was homogenized just before use in a microfuge tube at 4°C using a homogenizer fitting the tube at full speed for 1 minute after adding 20 mg insoluble polyvinylpyrrolidone in 0.2 ml of extraction buffer (100 mM Tris-HCl (pH 8.3), 50% v/v ethylene glycol, 140 mM ammonium sulphate, 56 mM 2-mercaptoethanol, 20 mM sodium bisulphate, 10 mM EDTA, 4 mM phenylmethylsulphonyl fluoride, 4 mM iodoacetamide), which was adjusted to 1 $\mu g\ ml^{-1}$ pepstatin A, 2 $\mu g\ ml^{-1}$ aprotinin and 2 $\mu g\ ml^{-1}$ leupeptin. The homogenate was centrifuged at 0°C for 15 min at 18,000*g* in a microfuge. The supernatant was mixed directly with 2 x standard concentration SDS-sample buffer (Laemmli 1970) and dissolved at 100°C for 2 min. Then 5 µl was immediately used for the SDS-PAGE and the remainder was stored at -20°C for further analysis.

About 0.5 g of frozen leaves were homogenized after adding 50 mg insoluble polyvinylpyrrolidone in 0.5 ml of extraction buffer, using a blender (Physcotron, Niti-on Co, Tokyo) at full speed for 1 min. The homogenate was centrifuged at 0°C for 15 min at 15,000*g*. The supernatant was collected and polyethylenimine was added to a final concentration of 0.1%. The extract was vortexed and centrifugated for 10 min at 12,000*g*. The supernatant was precipitated by adding 0.725 volumes of saturated ammonium sulphate solution. The precipitate was collected by centrifugation at 12,000*g* for 15 min, directly resuspended into SDS-sample buffer, and dissolved at 100°C for 2 min, 4 µl was directly used for the SDS-PAGE and the remainder was stored at -20°C for further analysis.

Proteins were electrophoresed in 6.5% SDS-polyacrylamide gels, using prestained molecular mass standards (SDS-7B markers, Sigma, St. Louis, MO). The apparent molecular mass of these prestained markers was recalibrated using high molecular mass standards (SDS-6H markers, Sigma) and then electroblotted onto a nylon filter (FineBlott; Atto, Tokyo) in 100 mM Tris-HCl, 192 mM glycine, and 20% (v/v) methanol. The membranes were blocked in a series of Tris-HCl buffer-saline-Tween (TBST) solutions, all containing 20 mM Tris-HCl, pH 7.5, and varying Tween-20 and NaCl concentrations: 2% (v/v) Tween and 500 mM NaCl for 3 min; 0.05% (v/v) Tween and 500 mM NaCl for 10 min; 0.05% (v/v) Tween and 150 mM NaCl for 3 min. Incubation with the primary antibody was in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% (w/v) fat-free milk powder. The monoclonal anti-PHYA and anti-PHYB antibodies used were mAP5 (Nagatani et al., 1985) and mAT1 (López-Juez et al., 1992) in dilutions of 2 $\mu g\ ml^{-1}$ and a 1:1 dilution of hybridoma culture supernatant, respectively. The incubation was at room temperature for 2 h, after washing three times with TBST, as at the end of the blocking, and membranes were incubated with a 1:5000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Protoblot kit; Promega Corp., Madison, WI) for 45 min, washed, and stained for alkaline phosphatase according to the manufacturer's instructions.

## Pretreatment of the seeds

To obtain a higher germination percentage, the seeds used in the EODFR, pulse, delayed R and broad-band light experiments, as well as those used for genetic analysis, were pretreated before the final sowing. The seeds were therefore sown in 9 x 9-cm plastic boxes on one layer of thick, absorbent paper (T300-45mm, Schut BV, Heelsum, The Netherlands) moistened with 7.4 ml germination buffer (0.01 M $NaH_2PO_4.H_2O$, 0.01 M $K_2HPO_4.3H_2O$, 5 mM $KNO_3$, pH 7.5) and placed in a darkroom at 25°C. After 2 days pretreatment, the seeds were planted out under dim-green safelight in trays filled with a mixture of potting compost and sand (volume ratio 3:1).

## Continuous broad-band light experiment

Pretreated seeds sown in trays were incubated in D for 72 h at 25°C. The irradiation with continuous B, R and FR (3 μmol $m^{-2}$ $s^{-1}$) was started just before the seedlings emerged through the soil surface. The length of 20 hypocotyls was measured daily for 7 days with a ruler under dim-green safelight. In addition, the hypocotyl length of seedlings grown in D was also measured daily. At the end of the experiment, the seedlings (four replicates) were used for the determination of anthocyanin in hypocotyls and chlorophyll in cotyledons and for the measurement of cotyledon area.

For the determination of anthocyanin, samples of five hypocotyls were extracted with 1.2 ml acidified (1% [w/v] HCl) methanol for 24 h in D with shaking. A Folch partitioning (Folch et al. 1957) was performed by adding 0.9 ml $H_2O$ and 2.4 ml of chloroform to the extracts and centrifugation for 30 min at 3,600 rpm. The absorbance of the top phase was determined with a Beckman DU-64 spectrophotometer (Beckman Instruments Inc, Fullerton, CA) at 535 nm.

The cotyledon area was measured with a leaf-analysis system (Skye Instruments Ltd, Powys, UK). For chlorophyll extraction, samples of 10 cotyledons were weighed, placed in glass tubes, immersed in a 100 times excess volume of *N,N*-Dimethylformamide (w/v) (Moran 1982), and incubated in D for 24 h. The absorbance of the extracts was measured at 647 nm and 664 nm and chlorophyll content on a fresh weight basis was calculated using the equations published by Inskeep and Bloom (1985).

## Pulse experiment

Pretreated seeds sown in trays were incubated in D for 48 h at 25°C. Pulses of R (3 min, 10 μmol $m^{-2}$ $s^{-1}$) or R immediately followed by FR (6 min, 13 μmol $m^{-2}$ $s^{-1}$), both saturating for attaining phytochrome photoequilibrium, were given every 4 h beginning at the time of emergence of the first seedlings. During the pulse irradiation, every seedling was marked on emergence, enabling the measurement of hypocotyl growth of each

seedling after the appropriate number of pulses (6, 12, 18 or 24). For each treatment 15-30 seedlings were measured.

## Delayed R experiment

Pretreated seeds sown in trays were either placed in D or in a continuous R cabinet at 25°C. After 72 h all seedlings that had just emerged were marked (day 1) and measured daily for 7 days with a ruler under dim-green safelight. In addition, after the measurement of seedlings grown in D at day 1 and day 3, some were transferred to continuous R (1d D → R; 3d D → R). For each treatment 10-25 seedlings were measured.

## End-of-day far-red light experiment

Pretreated seeds sown in trays were grown for 12 days in a phytotron with a daily irradiation schedule of 16 h of WL (PAR, 190 μmol $m^{-2}$ $s^{-1}$)/8 h of D at 25°C and relative humidity of 65 to 70%. At day 13 the seedlings were transplanted into 10-cm square plastic pots, and after transfer to cabinets at day 16, allowed to adjust to the lower level of WL (PAR, 125 μmol $m^{-2}$ $s^{-1}$) for 1 day before start of the experimental treatment. Plants were then selected for uniform height, and after the daily WL period received an immediate 20 min FR irradiation (4.6 μmol $m^{-2}$ $s^{-1}$). The controls were grown in a similar cabinet and received no FR irradiation. Plant height (six plants per treatment) was measured during a 15 day EODFR treatment.

## Supplementary daytime far-red light experiment

Seedlings of the *tri*$^{l}$ mutant and its isogenic WT GT were raised from seed at 25°C in a potting compost/sand mixture in 16 h WL (PAR, 170 μmol $m^{-2}$ $s^{-1}$)/8 h D cycle for 7 days. The plants were then transplanted into 10-cm diameter pots and transferred to a cabinet with the same cycle but higher irradiance (PAR, 250 μmol $m^{-2}$ $s^{-1}$) that had a R:FR photon ratio of 6.90. After 18 days the plants were transferred to two cabinets with a similar light/dark cycle, one of which had additional FR, which is not photosynthetically active and reduces the R:FR photon ratio to 0.13. All other environmental conditions within the cabinets were identical, with a 16 h photoperiod at a constant temperature of 25°C day/night and relative humidity of 70%. Plant height (six plants per treatment) was measured during a 6 day period of light treatment.

## Light sources

The broad-band B, R and FR cabinets used for the screening of mutants, the broad-band and delayed-R experiments, and genetic analysis were the same as described by Koornneef et al. (1980).

For the pulse experiment, R was obtained from light-emitting diodes (NLS01, 660 nm peak, half bandwidth 25 nm, Nijssen Light Division BV, Wageningen, The Netherlands), whereas the FR source was the same as that described by Koornneef et al. (1980).

The EODFR experiment was carried out in cabinets earlier described by Joustra (1970). WL was obtained from Philips TL40/33 fluorescent tubes. FR was provided by Sylvania F48T12/232/VHO tubes wrapped with one layer of dark-green and one layer primary-red filter (Lee, Flashlight Sales BV, Utrecht, The Netherlands).

The fluence rates and exposure times used are given in the description of each experiment. All light measurements were made using a LI1800/12 spectroradiometer (Li-cor, Lincoln, NE).

**Acknowledgements** This research was supported by grants to A.v.T. and L.H.J.K. from the Foundation for Life Sciences (SLW) formerly the Foundation for Biological Research (BION) which is subsidized by the Netherlands Organization for Scientific Research (NWO). We thank: E. López-Juez for help with Western blotting; Corrie Hanhart and Mariëlle Schreuder for technical assistance; S. Yoshida for use of greenhouse facilities at RIKEN; H. Smith and G.C. Whitelam, University of Leicester for the facilities used in the supplementary daytime FR experiments.

# Chapter 4

# Analysis of phytochrome-deficient *yellow-green-2* and *aurea* mutants of tomato

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Published in *Plant J* (1996) **9**: 173-182

**Abstract** *Two alleles of the* yellow-green-2 *(*yg-2*) and eight different alleles of the* aurea *(*au*) locus of tomato (*Lycopersicon esculentum *Mill.) were compared. All are characterized by a paler green colour compared to wild type (WT), an elongated hypocotyl in red light, and low or below detection limits of spectrophotometrically active phytochrome. Hypocotyl length was variable in white light, ranging from that of WT to more elongated. Immunochemical analysis revealed that etiolated seedlings of the* yg-2 *mutant have approximately 25% of the WT level of phytochrome A protein (PHYA), whereas that of phytochrome B protein (PHYB) is normal. In this it resembles the* au *mutant. The* au,yg-2 *double mutant has a more extreme chlorophyll deficiency than either parent. Since the* yg-2 *and* au *mutants have a less severe phenotype at the adult stage, that is are leaky, the additive effect can be explained by assuming that the mutants control two steps in the chromophore biosynthesis pathway. Combination, by crossing, of the* yg-2 *and* au *mutants with a transgenic tomato line that overexpresses oat phytochrome A3 (PhyA-3) essentially failed to restore the WT phenotype under white fluorescent light conditions, although under greenhouse conditions some evidence for increased sensitivity to light was observed. Immunochemically, oat PHYA-3 protein is detectable in both the* yg-2,PhyA-3 *and* au,PhyA-3 *'double' mutants. Spectrophotometrical analysis, however, revealed that holophytochrome was undetectable in the* yg-2,PhyA-3 *and* au,PhyA-3 *'double' mutants. These results are compatible with both mutants being disturbed in phytochrome chromophore biosynthesis.*

## Introduction

The perception of light by plants is mediated by different groups of photoreceptors: those that absorb in the UV-B, blue light (B)/UV-A and red light (R)/far-red light (FR). Of these photoreceptors the latter group, called phytochromes, is the best characterized and plays a role in seed germination, de-etiolation (inhibition of hypocotyl growth, opening of the apical hook, expansion of the cotyledons, development of chloroplasts, accumulation of

anthocyanin), shade avoidance and the induction of flowering (Kendrick and Kronenberg 1994).

The phytochrome molecule is a chromoprotein composed of an apoprotein (PHY) to which a linear tetrapyrrole chromophore is covalently attached (Terry et al. 1993), which exists as a homodimer. In higher plants phytochrome apoproteins are encoded by a small gene family, resulting in different types of phytochrome that vary in their temporal and spatial distribution, stability in the light and which probably have some unique functions. In tomato (*Lycopersicon esculentum* Mill.) at least six different phytochrome genes (*Phy*) have been identified (Hauser et al. 1994; Pratt 1995).

Mutants defective in phytochrome or the phytochrome transduction chain(s) are expected to have an altered response, especially to R and FR. The first mutants of this type described in tomato were *aurea* (*au*) and *yellow-green-2* (*yg-2*) (Koornneef et al. 1985). These mutants were initially selected for or identified by their pale-green colour, but in addition had an elongated hypocotyl and reduced anthocyanin content in white light (WL) (Koornneef et al. 1985). Both mutants had strongly reduced levels of spectrophotometrically detectable phytochrome (Koornneef et al. 1985). The *au* mutants have been studied extensively to: understand the molecular nature of the lesions involved (Parks et al. 1987; Sharma et al. 1993; Sharrock et al. 1988); examine the effect of phytochrome deficiency on gene expression (Becker et al. 1992; Oelmüller and Kendrick 1991; Oelmüller et al. 1989) and physiology (Adamse et al. 1988) and recently to study phytochrome signal transduction (Bowler and Chua 1994).

The absence of functional phytochrome A (phyA) in dark-grown seedlings (Koornneef et al. 1985; Parks et al. 1987) and the presence of an end-of-day FR (EODFR) response (Adamse et al. 1988), which is commonly accepted to be regulated by phytochrome B (phyB) (Adamse et al. 1987; Devlin et al. 1992; López-Juez et al. 1992; Reed et al. 1993), led to the conclusion that the *au* mutant might be specifically phyA deficient. The fact that Sharrock et al. (1988), using a RFLP for a phytochrome-coding

sequence from *Arabidopsis*, which they presumed was *PhyA*, mapped this gene to chromosome 10, distinct from the *au* locus, which maps on chromosome 1 (Balint-Kurti et al. 1995; Khush and Rick 1968; Soost and Lesley 1959) gave us reason to doubt that the *au* mutant is specific for phyA. The same argument applies to the *yg-2* locus on chromosome 12 (Kerr 1979c). Furthermore Sharma et al. (1993) showed that the PHYA apoprotein, although reduced, is present in the *au* mutant. Our doubts have been confirmed by the recent isolation of the phyA-deficient *fri* mutants of tomato (Van Tuinen et al. 1995a) which resemble the *phyA* mutants of *Arabidopsis* (Dehesh et al. 1993; Nagatani et al. 1993; Parks and Quail 1993; Whitelam et al. 1993) and have a phenotype clearly distinguishable from that of *au* and *yg-2*. In addition, the *fri* locus maps to chromosome 10 (Van Tuinen et al. 1995a), at a position similar to the *phyA* gene on the RFLP map (A. van Tuinen, unpublished data).

Parks and Quail (1991) concluded that the *hy1* and *hy2* mutants of *Arabidopsis* were chromophore mutants, because they were able to restore the wild-type (WT) phenotype for hypocotyl elongation and spectrally detectable phytochrome by feeding them the chromophore precursor biliverdin. This procedure also worked for the *pew1* mutant of *Nicotiana plumbaginifolia* (Kraepiel et al. 1994). The *pew2* mutant, however, could not be rescued by feeding biliverdin, which is also the case for the *au* mutant of tomato. In tomato this is probably due to technical problems. B.M. Parks and R. Sharma (personal communications) both tried to block the endogenous chromophore biosynthesis with gabaculine and subsequently failed to rescue the WT phenotype with biliverdin. Therefore it is possible that tomato has difficulty in taking up biliverdin, but it could also indicate that the block might be at a step in the pathway of phytochromobilin synthesis after biliverdin (Terry et al. 1993).

A way to show that a mutant is defective in a specific protein is to complement the mutation by transformation of the mutant plant with a WT gene encoding that protein. The overexpression of phytochrome in WT plants led to phenotypes characterized by a dwarf stature, increased chlorophyll

(Cherry et al. 1991) and anthocyanin content (Boylan and Quail 1989), that is, opposite to that of the *yg-2* and *au* mutants. Since the overexpressed phytochrome protein requires a chromophore, from the plant in which it is expressed, to form functional phytochrome, chromophore deficiency will lead to the absence of functional phytochrome, despite the fact that the protein is present.

In this paper different *au* and *yg-2* alleles along with the *au,yg-2* double mutant are compared. Attempts to restore the WT phenotype to the *yg-2* and *au* mutants by crossing in overexpression of the *PhyA-3* gene from oat are reported. The advantage of the hybridization approach rather than transforming the individual mutants with the gene construct directly, is that by this procedure no variation in expression of the transgene will complicate the comparison. Furthermore, problems were encountered by McCormac (1993) with the expression of the oat PhyA-3 when transformed directly into the *au* mutant. Although a few transformants were recovered that produced oat PhyA-3 mRNA, no oat PHYA-3 protein could be detected.

## Results and discussion

### Genetic and phenotypic characterization of *yg-2* and *au* and their double mutant

*Phenotype of different alleles of the* yg-2 *and* au *mutants*

The yellow leaf colour, which correlates with reduced chlorophyll content (Table 1), has been the main diagnostic characteristic for isolation or identification of the *au* and *yg-2* mutants. Additional common characteristics are a long hypocotyl, especially in R and FR, and a reduced phytochrome content (Table 1). The latter two characteristics indicate that phytochrome deficiency is the primary defect. Table 1 shows that, whereas the WTs hardly differ in their response in hypocotyl elongation to WL, the response among the *au*

**Table 1.** Comparison of *aurea* (*au*) and *yellow-green-2* (*yg-2*) alleles and their corresponding WT

| Genotype | Hypocotyl length ± SE | | | Chlorophyll (μg/gFw) ± SE | Ptot $10^3$ $\Delta(\Delta A_{660-730})$ |
|---|---|---|---|---|---|
| | D | R | WL | | |
| CR | 148 ± 3 | 42 ± 1 | 32 ± 0 | 1204 ± 26 | 19.5 |
| $au^{fs}$ | 167 ± 3 | 160 ± 2 | 47 ± 1 | 405 ± 12 | 0.0 |
| VF145 | 174 ± 3 | 62 ± 1 | 33 ± 1 | 1169 ± 23 | 18.4 |
| $au^{tl}$ | 191 ± 4 | 176 ± 2 | 48 ± 1 | 568 ± 12 | 0.0 |
| UC105 | 161 ± 4 | 59 ± 1 | 39 ± 1 | 1234 ± 16 | 22.3 |
| $au^{pi}$ | 188 ± 2 | 164 ± 2 | 69 ± 1 | 343 ± 15 | 0.0 |
| Z0 | 162 ± 2 | 53 ± 1 | 35 ± 1 | 1179 ± 23 | 25.5 |
| $au^{pm}$ | 153 ± 4 | 127 ± 3 | 41 ± 1 | 672 ± 30 | 0.1 |
| $au^{b}$ | 163 ± 3 | 118 ± 2 | 38 ± 1 | 693 ± 23 | 0.6 |
| MM | 166 ± 2 | 56 ± 1 | 38 ± 1 | 1156 ± 25 | 17.8 |
| $au^{w}$ | 183 ± 2 | 175 ± 2 | 66 ± 1 | 499 ± 30 | 0.0 |
| AC | 181 ± 2 | 55 ± 1 | 33 ± 1 | 1377 ± 61 | 19.5 |
| *au* | 185 ± 3 | 163 ± 3 | 56 ± 1 | 516 ± 27 | 0.1 |
| $au^{6}$ | 130 ± 8 | 133 ± 5 | 51 ± 2 | 585 ± 37 | 0.0 |
| $yg\text{-}2^{aud}$ | 139 ± 6 | 130 ± 4 | 43 ± 1 | 280 ± 8 | 1.0 |
| *yg-2* | 201 ± 3 | 178 ± 2 | 60 ± 1 | 541 ± 19 | 1.0 |

Hypocotyl length was measured after 7 days continuous dark (D), red (R, 3.5 μmol $m^{-2}$ $s^{-1}$) or a 16 h white light (WL, photosynthetically active radiation 150 μmol $m^{-2}$ $s^{-1}$)/8 h D regime. Chlorophyll content was measured after 7 days WL/D regime as described above. Total phytochrome (Ptot) content was determined in dark-grown 4-day-old seedlings using a dual-wavelength spectrophotometer and is expressed as Δ(ΔA)/40 hypocotyl sections (mean of two samples).

alleles varies from being similar to, to almost twice the length of the WT. For the $au^b$ this seems to be related to the presence of a low, yet detectable, amount of residual phytochrome. However, despite its more extreme leaf colour and low level of spectrophotometrically detectable phytochrome, the hypocotyl of the $yg\text{-}2^{aud}$ allele in WL is only slightly longer than that of the WT (Table 1), confirming the observation of Rick et al. (1968). The reduced chlorophyll content of the $yg\text{-}2^{aud}$ allele is exceptional, since it is much lower than that of the other *yg-2* allele and all *au* alleles. The most likely explanation would be that $yg\text{-}2^{aud}$ is an extreme allele of the *yg-2* locus. This, however, does not agree with the fact that the phytochrome content is the same in both *yg-2* alleles, suggesting that the extreme chlorophyll deficiency is specific for the $yg\text{-}2^{aud}$ allele.

The phytochrome protein levels of the *yg-2* mutant are shown in Figure 1. The PHYB protein is present at the WT level, whereas the PHYA protein level is reduced to approximately 25% of the WT. This is the same as the situation reported previously for the *au* mutant (Sharma et al. 1993).

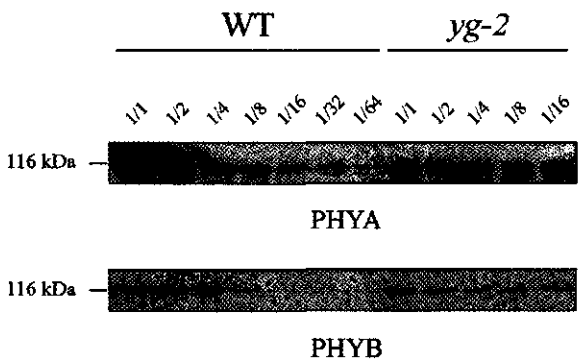


**Figure 1.** Immunologically detectable phytochrome in wild-type and *yellow-green-2* (*yg-2*) mutant seedlings. Serial dilutions of extracts containing phytochrome A and B polypeptides (PHYA and PHYB, respectively) of wild-type (WT, cv. MM) and *yg-2* mutant seedlings. Dark-grown 4-day-old seedlings were used for the detection of PHYA and PHYB with monoclonal antibodies mAP5 and mAT1, respectively, in crude extracts.

*Phenotype of the* au,yg-2 *double mutant*

Chiscon (1960b) briefly mentioned that the leaves of a double mutant between *yg-2* and $au^6$ had yellow-white patches and measured a slightly reduced chlorophyll content in them compared with those of both single mutants (Chiscon 1961). However, no additional data on plant vigour and genetic characterization were given. The $F_2$ generation derived from the cross between W616 ($au^w$) and LA1171 ($yg\text{-}2^{aud}$) was studied to determine the interaction between *au* and *yg-2*. In addition to WT seedlings and seedlings with parental phenotypes, a small fraction of seedlings with a more extreme deficiency in de-etiolation than that of either single parent, that is, yellow-white cotyledons (Fig. 2), was observed. The majority of these seedlings died after development of a few true leaves, which were almost white under greenhouse conditions. The few surviving seedlings slowly started to green and developed into delicate, retarded plants, with yellow leaves, but ultimately some seed set was obtained. Backcrossing of these plants with both monogenic parents revealed that they were homozygous recessive for both *au* and *yg-2*. The additive effect of the two mutations might suggest that they control similar parallel pathways. However, it is also possible that the mutants are somewhat 'leaky'. In such a case genes controlling subsequent steps in a sequential pathway are also expected to have additive effects. Leakiness of the *au* and *yg-2* phenotypes can be inferred from the observation that the de-etiolated phenotype of these mutants at the adult stage is less severe than at the seedling stage, which is also observed for the double mutant. Furthermore, the ability to measure spectrophotometrically active phytochrome in flowers (Adamse et al. 1988) and in extracts from adult leaves of the *au* mutant (Sharma et al. 1993) is also compatible with leakiness. An additive effect of two putative chromophore mutants resulting in a seedling lethal has been described by Kraepiel et al. (1994). The lack of hypocotyl elongation in the *au,yg-2* double mutant, in contrast to the extremely elongated hypocotyl of the *pew1,pew2* double mutant of *N. plumbaginifolia* (Kraepiel et al. 1994), can be explained by the fact that the $yg\text{-}2^{aud}$ allele is epistatic over *au* for hypocotyl elongation, but possible

photosynthetic limitations due to severe photobleaching in the double mutant could confuse the situation. The *au,yg-2* double mutant has precisely the same amount of immunologically detectable PHYA as the monogenic mutant parent lines, that is, there is no additive effect (data not shown). This is consistent with the reduced level of protein being a consequence of the unavailability of the chromophore. This contrasts with the situation in the *hy1* and *hy2* mutants of *Arabidopsis* (Parks and Quail 1991) and the *pcd1* mutant of pea (Weller et al. 1996) where the PHYA protein appears to be stable.


**Figure 2.** Phenotypic comparison of genotypes in white light. Seedlings of wild-type (WT1, cv. MM; WT2, cv. AC), the *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutants and the *au,yg-2* double mutant grown for 28 days in a Dutch greenhouse in winter with a day/night temperature regime of 21°/15°C.

## The phenotype and phytochrome analysis of *yg-2* and *au* mutants overexpressing the oat *PhyA-3* gene

To test whether the mutant phenotype of the *yg-2* and *au* could be restored to WT by overexpressing the phyA protein we crossed both mutants with a transgenic tomato line overexpressing oat PhyA-3 (Boylan and Quail 1989). Five-day-old $F_2$ seedlings from the cross between *yg-2* and the oat PhyA-3 overexpressor were scored for hypocotyl colour after a 24 h R treatment. Seedlings with purple (62), grey (24) and white (16) hypocotyls developed as expected into plants with overexpressor (short with dark-green leaves), WT and *yg-2* (elongated with pale-green leaves) phenotypes, respectively. This segregation suggests that the dominant PhyA overexpressor phenotype is not expressed in the *yg-2* mutant background, otherwise *yg-2* mutants would be expected in a frequency of only 1/16. The $F_2$ population subsequently was checked on a larger scale on medium containing kanamycin to identify plants carrying the transgene. In this test system the $F_2$ generation derived from the crosses between the oat PhyA-3 overexpressor and the *au* and *yg-2* mutants both segregated in a 9:3:3:1 ratio of short, kanamycin-resistant: normal, kanamycin-sensitive: tall, kanamycin-resistant: tall, kanamycin-sensitive seedlings, respectively, ($\chi^2_{yg\text{-}2} = 3.69$; $\chi^2_{au} = 8.06$; $0.01 < P < 0.05$) expected for two independent loci (Fig. 3). The high $\chi^2$ value for the *au* $F_2$ is caused by a shortage of *au* mutant seedlings, as a result of the poor germination of the *au* mutant (Georghiou and Kendrick 1991; Koornneef et al. 1985). Figure 4 shows that the hypocotyl length of seedlings of the oat PhyA-3 overexpressor and an $F_3$ line of the 'double' mutant (homozygous for both *yg-2* and oat *PhyA-3*) is the same on medium with or without kanamycin. The hypocotyl length of WT and *yg-2* mutant seedlings, however, is inhibited on medium containing kanamycin. Similar results were obtained for the *au* mutant. These data indicate that in *yg-2* and *au* backgrounds no phenotypic effect of the *PhyA-3* transgene is observed at the seedling level when they are grown under fluorescent WL.

| | | | | | |
|---|---|---|---|---|---|
| P | genotype: | *M/M, PhyA-3/PhyA-3* x | *m/m, -/-* | | |
| | phenotype: | dwarf$^{res}$ | long hypocotyl$^{sen}$ | | |
| $F_1$ | genotype: | *M/m, PhyA-3/-* | | | |
| | phenotype: | dwarf$^{res}$ | | | |
| $F_2$ | genotype: | *M/., PhyA-3/.* | *M/., -/-* | *m/m, PhyA-3/.* | *m/m, -/-* |
| | phenotype: | dwarf$^{res}$ | wild type$^{sen}$ | long hypocotyl$^{res}$ | long hypocotyl$^{sen}$ |
| | segregation: *au* | 192 | 70 | 41 | 21 |
| | *yg-2* | 253 | 79 | 68 | 21 |

**Figure 3.** Genetic segregation of *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutant phenotypes and oat *PhyA-3* overexpression. Scheme describing the isolation of the 'double' mutants between the *au* or *yg-2* mutant (m) and the oat PhyA-3 overexpressing transgenic tomato line (*PhyA-3*). Seedlings were selected on medium containing kanamycin and expected to be either resistant (res) or sensitive (sen).

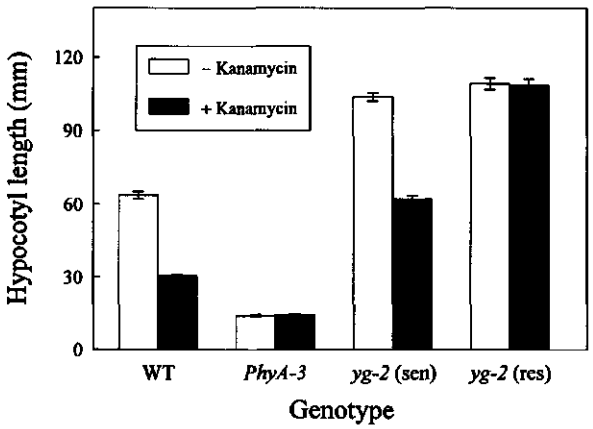


**Figure 4.** Effect of kanamycin on hypocotyl growth of wild-type and mutant seedlings. Hypocotyl length of tomato wild-type (WT, cv. MM), oat PhyA-3 overexpressor (*PhyA-3*), *yellow-green-2* mutant (kanamycin sensitive, *yg-2*(sen)) and *yg-2,PhyA-3* 'double' mutant (kanamycin resistant, *yg-2*(res)) seedlings measured after 14 days growth on medium with (*filled bars*) or without (*open bars*) 100 mg $l^{-1}$ kanamycin. Error bars represent the SE of the means.

Although not visible when grown in tissue culture containers there is some evidence for rescue of hypocotyl length in the *au,PhyA-3* 'double' mutant when grown in soil under phytotron conditions (Fig. 5). This slight difference in length remains during further development (Fig. 6). However, seedlings of the same genotypes grown in a greenhouse in Japan during winter in all cases had a much longer hypocotyl and the rescue of the *au,PhyA-3* 'double' mutant was more obvious (Fig. 5). Results for the *yg-2,PhyA-3* 'double' mutant were similar, although less pronounced (results not shown).

In extracts of etiolated seedlings of the oat PhyA-3 overexpressor and its isogenic WT the pea phyA monoclonal antibody (mAP5) recognizes the 116 kDa protein of tomato, but also cross hybridizes slightly with the 124 kDa oat PHYA-3 polypeptide in the transgenic line (Fig. 7). However, the mAR14 antibody raised against rye phyA recognizes only the oat PHYA-3 protein in the transgenic tomato line (Fig. 7). The presence of an immunochemically detectable pool of the 124 kDa PHYA-3 protein in the *yg-2,PhyA-3* 'double' mutant proves that the oat *PhyA-3* gene is expressed and PHYA-3 protein is produced in similar amounts as in the overexpressing parent line. In both WTs and the PhyA-3 overexpressor the tomato PHYA protein disappears after 4 h R treatment, whereas the level of PHYA protein in the *yg-2* mutant and the *yg-2,PhyA-3* 'double' mutant is unchanged after this treatment (Fig. 8). The lack of degradation of the immunochemically detectable tomato PHYA in R in the *yg-2* mutant and the *yg-2,PhyA-3* 'double' mutant suggests that this PHYA is not spectrally active. The level of a PHYB-like protein, detected with mAT1, in the *yg-2* mutant, the *yg-2,PhyA-3* 'double' mutant and the PhyA-3 overexpressing line is comparable to that in the WTs (Fig. 8). Similar results were obtained for the *au* mutant and *au,PhyA-3* 'double' mutant (data not shown).

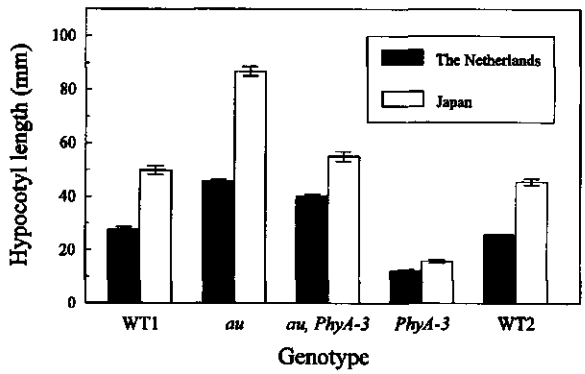


**Figure 5.** Influence of light environment on hypocotyl growth inhibition. Hypocotyl length of tomato wild-type (WT1, cv. MM; WT2, cv. VF36), *aurea* mutant (*au*), oat PhyA-3 overexpressor (*PhyA-3*) and *au,PhyA-3* 'double' mutant seedlings measured after 5 days in a 16 h WL (photosynthetically active radiation 160 μmol $m^{-2}$ $s^{-1}$)/8 h dark cycle at 25°C in a phytotron in The Netherlands (*filled bars*) or 28 days in a Japanese greenhouse (*open bars*). Error bars represent the SE of the means.


**Figure 6.** Phenotypic comparison of genotypes in white light. Wild-type (WT, cv. VF36), oat PhyA-3 overexpressor (*PhyA-3*), *aurea* (*au*),*PhyA-3* 'double' mutant and *au* mutant plants grown for 1 month in a 16 h WL (photosynthetically active radiation 160 μmol $m^{-2}$ $s^{-1}$)/8 h dark cycle at 25°C.

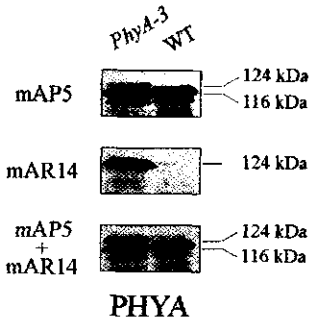


**Figure 7.** Immunological detection of tomato and oat phytochromes in wild-type and oat phytochrome A-3 overexpressing tomato lines. Tomato and oat phytochrome A polypeptide (PHYA) were detected with monoclonal antibodies mAP5 and mAR14, respectively, in crude extracts of 4-day-old dark-grown wild-type (WT, cv. VF36) and oat PhyA-3 overexpressing (*PhyA-3*) seedlings.

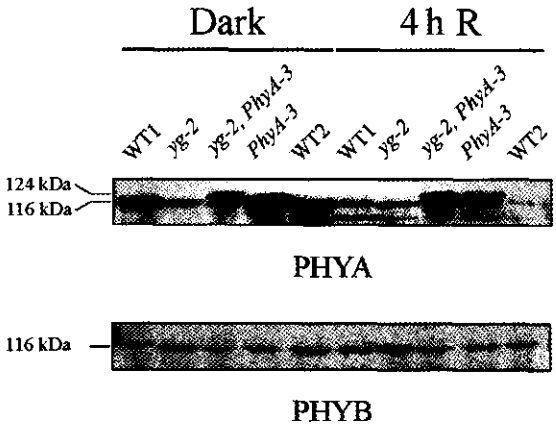


**Figure 8.** Phytochrome content of different genotypes after dark or red light treatment. Immunoblot detection of phytochrome A and B polypeptide (PHYA and PHYB, respectively) in wild-type (WT1, cv. MM; WT2, cv. VF36), oat PhyA-3 overexpressor (*PhyA-3*), *yellow-green-2* (*yg-2*) mutant and *yg-2,PhyA-3* 'double' mutant seedlings. Dark-grown 4-day-old seedlings or seedlings of the same age exposed to 4 h of red light (R) were used for the detection of PHYA and PHYB with a mixture of monoclonal antibodies of mAP5 and mAR14 or mAT1, respectively, in crude extracts.

Spectrophotometric analysis (Fig. 9) demonstrates that overexpression of oat PHYA-3 results in a two-fold increase in spectrophotometrically measurable phytochrome in etiolated seedlings. Although both tomato and oat PHYA-3 protein are present in the *au,PhyA-3* 'double' mutant there is no spectrophotometrically detectable phytochrome. In the *yg-2* mutant and the *yg-2,PhyA-3* 'double' mutant, however, a trace of spectrophotometrically active phytochrome is measured (Table 1, Fig. 9). Spectrophotometric analysis showed that the light-labile pool in tomato is more or less depleted after 4 h R (Van Tuinen et al. 1995a). The difference in phytochrome content between the oat PhyA-3 overexpressor and its isogenic WT must therefore be due to oat PHYA-3 protein. This result is in agreement with earlier observations that the introduced oat phytochrome pool degrades more slowly than endogenous dicotyledonous phyA (Boylan and Quail 1989, 1991; Cherry et al. 1991; McCormac et al. 1992).

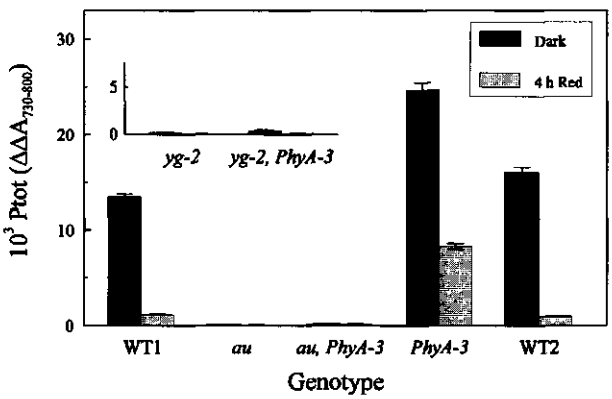


**Figure 9.** Spectrophotometric measurement of phytochrome in seedlings. *In vivo* measurement of spectral activity of phytochrome in wild-type (WT1, cv. MM; WT2, cv. VF36), oat PhyA-3 overexpressor (*PhyA-3*), *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutant, *au,PhyA-3* and *yg-2,PhyA-3* 'double' mutant seedlings. The total phytochrome (Ptot) content was measured in dark-grown 4-day-old seedlings or seedlings of the same age exposed to 4 h of red light (R) using a dual-wavelength spectrophotometer and is expressed as $\Delta(\Delta A)/40$ hypocotyl sections. Error bars represent the SE of the means.

The WT seedlings grown in the greenhouse in Japan have hypocotyls much longer than those grown in the phytotron in The Netherlands (Fig. 5), presumably due to a combination of effects: the presence of FR in daylight and FR-rich reflected light from other plants resulting in a marked shade avoidance response; the low-light daylength extension. The shade avoidance response is thought to be mainly attributable to phyB and has already been demonstrated to be exhibited by the *au* mutant (Kerckhoffs et al. 1992). At the seedling stage, Quail et al. (1995) have pointed out the antagonism between phyA and phyB in the regulation of hypocotyl growth. In a situation where the chromophore is limiting it is clear that any accumulation of chromophore in PHYA will be lost by destruction, whereas any accumulation into stable phytochromes will be retained. We assume this must be the reason why the *au* mutant has its most pronounced phenotype at the hypocotyl stage. If the light levels are sufficiently high, as in the case of the WL in the phytotron, which is rich in B as well as R, the phenotypic effect of the *au* mutation on the hypocotyl length is less pronounced (Fig. 5). This presumably occurs by maintenance of a high Pfr level within the phyB pool which we believe is gradually filled during de-etiolation since the mutations are leaky, and activation of the B photoreceptor. Under conditions of natural daylight, particularly in the presence of FR-rich reflected light from other plants the FR-absorbing form of phytochrome (Pfr) level will be lower than in the phytotron and the shade avoidance response will be triggered resulting in a longer hypocotyl. Normally the contribution of phyA to the stature of an adult tomato plant is little, since the pool is rapidly exhausted in the light environment, a point substantiated by the phenotype of the phyA-deficient *fri* mutants (Van Tuinen et al. 1995a). However, if phyA were present under FR-rich conditions it would be expected to act antagonistically to the shade avoidance response. This is demonstrated clearly in the oat PhyA-3 overexpressing line, resulting in very short hypocotyls, even under conditions in which there is essentially no FR, that is, in the phytotron. In a situation where the 'double' mutants have long hypocotyls in the greenhouse in Japan, conditions exist which are extremely sensitive to test for the existence of any

photoactive oat phyA-3. We already have circumstantial evidence that very low levels of phytochrome can have an effect on the hypocotyl length, as indicated by the differences in severity of the phenotypes of the different *au* and *yg-2* alleles (Table 1). It is therefore possible that a trace of oat phyA-3 present in the 'double' mutants, which we know is more stable than any endogenous phyA (Boylan and Quail 1989), could result in the partial rescue of the WT phenotype under the greenhouse conditions. While this results in a hypocotyl length similar to that in the WT, it still falls far short of the hypocotyl length of the overexpressing oat phyA-3 parent line.

### The defects of the *yg-2* and *au* mutants

The results presented might suggest that the lack or reduced level of PHYA is the factor responsible for the lack of spectrophotometrically active phytochrome in the *yg-2* and *au* mutants. Mutants at the *fri* locus, however, contain less PHYA, but more spectrophotometrically detectable phytochrome than the *yg-2* and *au* mutants, because of the presence of other phytochromes (Van Tuinen et al. 1995a). This observation alone strongly suggests that at the seedling stage not only phyA, but also other phytochrome types are strongly reduced in both the *yg-2* and *au* mutants. A deficiency in availability of the common chromophore therefore seems to be the most likely consequence of the lesions in the *yg-2* and *au* mutants.

## Material and methods

### Plant material

The first *aurea* (*au*) mutant in tomato (*Lycopersicon esculentum* Mill.) was isolated by Lesley and Lesley (1956) from $^{32}P$-treated material. This stock was later used by Darby et al. (1978) to make a near-isogenic line of the cv. Ailsa Craig (AC). In 1957 Stubbe (Stubbe 1957) isolated the *bracteata* mutant in the progeny of X-ray-treated Condine Red

(CR) seeds. Because of the resemblance to *au*, Rick (1959) checked for allelism and found that *au* and *brac* were indeed allelic. The name *brac* was later changed to $au^{ls}$ (Anonymous 1973). The isolation of $au^{w}$ in cv. Moneymaker (MM) has been described by Koornneef et al. (1981, 1985). Rick and Zobel (1970) reported that the spontaneous chlorophyll mutant in VF145 background, sent to them by Torrey Lyon, is allelic to *au* and designated it $au^{ll}$. Two independently induced chlorophyll deficient mutants, C1 and D8, were isolated by Peter Morris at the IGF in Berlin in the $M_2$ population derived from seeds of breeding line Z0, which resembles AC in overall morphology, treated with EMS at the Department of Genetics, Wageningen Agricultural University. Allelism with *au* for both mutants was confirmed in Berlin as well as in Wageningen. We propose the gene symbols $au^{pm}$ (Peter Morris) and $au^{b}$ (Berlin) for C1 and D8, respectively. Burdick (1960) described $au^{6}$ initially as $yg_{389}$ (yo), an irradiation induced mutant in *L. esculentum* var. *cerasiformi* line 018. The name was later changed to *yg-6* (Burdick 1961). Koornneef et al. (1986) found that it was allelic to *au*. Another *au* allele was isolated from tissue culture experiments with cv. UC105 (Lipucci Di Paola et al. 1988). Whereas the allele has not yet been named, we propose the gene symbol $au^{pi}$ (Pisa) after the city of isolation. The original *yellow-green-2* (*yg-2*) mutant, initially named $yg_{282}$, was described by Burdick (1958) as a long-hypocotyl mutant with chlorotic yellow-green cotyledons and leaves, induced by irradiation of *L. pimpinellifolium* line 215. The second allele was initially described as *auroid* (*aud*) having bright yellow foliar parts but differing from *au* in not being elongate in the seedling stage (Rick et al. 1968). Allelism with *yg-2* was based on the lack of complementation of leaf colour by Kerr (1981).

For the construction of the *au,yg-2* double mutant $au^{w}$ was crossed with LA1171, a line obtained from Professor C.M. Rick (Tomato Genetics Resource Centre (TGRC), Davis, CA), which carries the *yg-2 auroid* allele ($yg\text{-}2^{aud}$). For the determination of hypocotyl length of the *au* and *yg-2* alleles, $yg\text{-}2^{aud}$ as a near-isogenic line of cv. AC (LA 3165) was used.

The homozygous transgenic tomato line, which overexpresses the oat PhyA-3 used was that obtained by transforming tomato cv. VF36 (LA490) with the construct pFY123 (Boylan and Quail 1989).

## Growth and selection of *yg-2* and *au* mutants overexpressing the oat *PhyA-3* gene

Seeds of selfed $F_1$ plants from the cross between *yg-2* and the oat PhyA-3 overexpressor were sown on thick absorbent paper (T300-45mm, Schut BV, Heelsum, The Netherlands) moistened with germination buffer (see pretreatment of the seeds), given a 72 h D period followed by a 24 h R treatment, after which the colour of the hypocotyl was scored. Seedlings were then transplanted in trays with potting compost and placed in a greenhouse (for conditions see section on growth in WL). Plants with *yg-2* or *PhyA-3* overexpressing phenotype were grown to maturity and $F_3$ seeds were harvested per plant.

The $F_3$ seeds were briefly immersed in 70% alcohol, surface sterilized with a 2% (v/v) solution of commercial bleach for 30 min and washed 3 times for 10 min with

sterile demineralized water. Per $F_3$ line 25 seeds were sown in plastic boxes on solidified medium according to Murashige and Skoog (1962) without hormones; 20 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar and 100 mg $l^{-1}$ filter-sterilized kanamycin were added. The boxes were placed in a growth chamber at 25°C and kept in darkness (D) for 6 days, after which they were transferred to a 16 h WL (photosynthetically active radiation [PAR, 400-700 nm], 50 µmol $m^{-2}$ $s^{-1}$; Philips TLD36/84)/8 h D regime. At day 20 after sowing the seedlings were scored for phenotype, that is, leaf colour, hypocotyl length and kanamycin resistance. Kanamycin-resistent seedlings are characterized by development of lateral roots and primary leaves and exhibit normal hypocotyl elongation. Lines that were homozygous for both the *yg-2* phenotype and kanamycin resistance were selected. The shoot tip of the seedlings was taken and placed on fresh medium. After 1 week, rooted seedlings were planted in small pots with potting compost and transferred to the greenhouse.

## Pretreatment of the seeds

To obtain a higher germination percentage the seeds used for growth in WL (Fig. 9) and for photography (Fig. 2) were pretreated before the final sowing. The seeds were therefore sown on thick absorbent paper in a plastic box (10 x 10 cm) or on two layers of filterpaper in a 9 cm petri dish moistened with 7.4 or 3 ml germination buffer (0.01 M $NaH_2PO_4.H_2O$, 0.01 M $K_2HPO_4.3H_2O$, 5 mM $KNO_3$, pH 7.5), respectively and placed in a darkroom at 25°C.

## Growth in WL

Pretreated seeds of WT (MM and AC), $au^w$, $yg\text{-}2^{aud}$ and the $au^w$,$yg\text{-}2^{aud}$ double mutant sown in trays filled with potting compost were placed in a greenhouse with a day/night temperature regime of 21°C/15°C. Photoperiod extension to 16 h WL was given with a mixture of high-pressure mercury (Philips HPI-T18, 400W) and sodium (Philips Son-T14, 400W) lamps. During the day lamps were switched off when the natural irradiation outside was equal to or greater than 24 mW $cm^{-2}$.

Pretreated seeds of WT (MM and VF36), the oat PhyA-3 overexpressor, the *au* and *yg-2* mutants and the *au,PhyA-3* and *yg-2,PhyA-3* 'double' mutants were sown in trays filled with a mixture of potting compost and sand (volume ratio 3:1) and placed in a phytotron at 25°C with a daily regime of 16 h WL (Philips TLD36/84; PAR 160 µmol $m^{-2}$ $s^{-1}$)/8 h D.

Pretreated seeds of WT (MM and VF36), the oat PhyA-3 overexpressor, the *au* and *yg-2* mutants and the *au,PhyA-3* and *yg-2,PhyA-3* 'double' mutants sown in trays filled with a mixture of granular clay-based compost and vermiculite (volume ratio 2:3) were placed in a greenhouse in Japan at 25°C. Photoperiod extension to 16 h WL was given with high-pressure mercury lamps (FLR 405.W/M/36 [Toshiba, Tokyo], PAR 10 µmol $m^{-2}$ $s^{-1}$).

## Experiment with all *au* and *yg-2* alleles

Pretreated seeds of all *yg-2* and *au* alleles and their corresponding WTs, except *L. pimpinellifolium* line 215 which was not available to us, were planted out in trays filled with a mixture of potting compost and sand (volume ratio 3:1) in WL or under dim green safelight and placed in a phytotron at 25°C with a daily regime of 16 h WL (Philips TLD36/84; PAR 150 μmol $m^{-2}$ $s^{-1}$)/8 h D or in a cabinet in D or with continuous R (Philips TL40/103339 filtered through one layer of primary red filter [Lee, Flashlight Sales BV, Utrecht, The Netherlands], 3.5 μmol $m^{-2}$ $s^{-1}$ ), respectively. Hypocotyl length and chlorophyll content were measured 7 days after emergence of the seedlings. For hypocotyl length at least 20 seedlings of each genotype per treatment were measured. Chlorophyll was extracted using a method adapted from that described by Hiscox and Israelstam (1979). Eight shoot tips (excluding cotyledons) per genotype were individually weighed, placed in glass tubes and incubated in D for 24 h at 65°C with 5 ml dimethyl sulphoxide (DMSO) and assayed immediately after cooling down to room temperature. Chlorophyll content was calculated using the equations for ethanol published by Lichtenthaler and Wellburn (1983).

## Growth of plants for phytochrome assays

Seeds were briefly surface sterilized under WL with a 1% (v/v) solution of commercial bleach for 3 min and then washed thoroughly with running tap water. Seeds were then sown on 0.6% (w/v) agar-medium containing 0.46 g $l^{-1}$ of Murashige-Skoog salts (Gibco. Gaithersburg, MD) in plant tissue culture containers obtained from Flow Laboratories (McLean, VA). Seedlings were grown at 25°C for 4 days either in D or irradiated with R (20 μmol $m^{-2}$ $s^{-1}$; white fluorescent tubes [FL20S.W.SDL.NU; National, Tokyo] filtered through 3-mm red acrylic [Shinkolite A102; Mitsibutsi Rayon, Tokyo]) for 4 h prior to harvest. The upper 1 cm of the hypocotyls, including the cotyledons were harvested under dim green safelight after gently removing any remaining seedcoats. For *in vivo* spectrophotometry the samples were collected on ice and used immediately. The samples for immunoblotting were frozen in liquid nitrogen and stored at -80°C before analysis.

## *In vivo* phytochrome spectrophotometry

For the spectrophotometric measurements of phytochrome, about 0.4 g tissue (collected from 40 seedlings) was gently packed into a custom-built stainless steel cuvette with glass windows (10 mm in diameter and about 4 mm path length) and the phytochrome content was measured in a dual-wavelength spectrophotometer (model 557; Hitachi, Tokyo) using 730-800 or 660-730 nm measuring beams, which was equipped with an actinic irradiation unit for photoconverting the sample with saturating irradiations of R (30 s) and FR (60 s).

## Phytochrome extraction and immunoblotting

About 0.2 g (collected from 20 seedlings) frozen material was homogenized after adding 20 mg insoluble polyvinylpyrrolidone in 0.2 ml extraction buffer (100 mM Tris-HCl (pH 8.3), 50% (v/v) ethylene glycol, 140 mM ammonium sulphate, 56 mM 2-mercaptoethanol, 20 mM sodium bisulphate, 10 mM EDTA, 4 mM phenylmethylsulphonyl fluoride, 4 mM iodoacetamide), which was adjusted to 1 $\mu g\ ml^{-1}$ pepstatin A, 2 $\mu g\ ml^{-1}$ aprotinin and 2 $\mu g\ ml^{-1}$ leupeptin just before use in a microfuge tube at 4°C, using an homogenizer fitting the tube at full speed for 1 min. The homogenate was centrifuged at 0°C for 15 min at 18,000*g* in a microfuge. The supernatant was mixed directly with 2 x standard concentration SDS-sample buffer (Laemmli 1970) and dissolved at 100°C for 2 min. Then 5 µl was immediately used for the SDS-PAGE and the remainder was stored at -20°C for further analysis.

Proteins were electrophoresed in 6.5% SDS-polyacrylamide gels, using prestained molecular mass standards (SDS-7B markers, Sigma, St. Louis, MO). The apparent molecular mass of these prestained markers was recalibrated using high molecular mass standards (SDS-6H markers, Sigma), and then electroblotted onto a nylon filter (FineBlott; Atto, Tokyo) in 100 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol. The membranes were blocked in a series of Tris-HCl buffer-saline Tween (TBST) solutions, all containing 20 mM Tris-HCl, pH 7.5, and varying Tween-20 and NaCl concentrations: 2% (v/v) Tween and 500 mM NaCl for 3 min; 0.05% (v/v) Tween and 500 mM NaCl for 10 min; 0.05% (v/v) Tween and 150 mM NaCl. Incubation with the primary antibody was in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1% (w/v) fat-free milk powder. The monoclonal anti-PHYA and anti-PHYB antibodies used were mAP5 (Nagatani et al. 1984), mAR14 (Nagatani et al. 1983) and mAT1 (López-Juez et al. 1992) in dilutions of 2 $\mu g\ ml^{-1}$ and a 1:1 dilution of hybridoma culture supernatant, respectively. The incubation was at room temperature for 2 h, after washing three times with TBST, as at the end of the blocking, membranes were incubated with a 1:5000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Protoblot kit; Promega Corp., Madison, WI) for 45 min, washed, and stained for alkaline phosphatase according to the manufacturers instructions.

**Acknowledgements** This research was supported by grants to A.v.T. and L.H.J.K. from the Foundation for Life Sciences (SLW) formerly the Foundation for Biological Research (BION) which is subsidized by the Netherlands Organization for Scientific Research (NWO). Research in the laboratory of P.H. Quail was supported by N.I.H. grant no. GM47475 and USDA/ARS CRIS grant no 5335-21000-006-00D. We thank: P. Morris for provision of the *aurea* alleles C1 and D8; C. Rick and R. Chetelat of the TGRC for supplement of several tomato genotypes; E. López-Juez for help with Western blotting; S. Yoshida for use of greenhouse facilities at RIKEN; K. Yoshizawa for technical assistance and M.J. Terry for comments on draft versions of the manuscript.

# Chapter 5

# The mapping of phytochrome genes and photomorphogenic mutants of tomato

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Accepted by Theor Appl Genet

**Abstract** *In tomato (*Lycopersicon esculentum *Mill.) the map positions of 5 previously described phytochrome genes have been determined. The position of the* yg-2 *gene on chromosome 12 has been confirmed and the classical map revised. The position of the phytochrome A (phyA)-deficient* fri *mutants has been refined by revising the classical map of chromosome 10. The position of the PhyA gene is indistinguishable from that of the fri locus. The putative phyB1-deficient* tri *mutants were mapped by classical and RFLP analysis to chromosome 1. The* PhyB1 *gene, as predicted, was located at the same position. Several mutants with the* high pigment *(*hp*) phenotype, which exaggerates phytochrome responses, have been reported. Allelism tests confirmed that the* hp-2 *mutant is not allelic to other previously described* hp *(proposed here to be called* hp-1*) mutants and a second stronger* hp-2 *allele (*hp-$2^j$*) was identified. The* hp-2 *gene was mapped to the classical, as well as the RFLP map of chromosome 1.*

# Introduction

Light plays a crucial role in the life cycle of plants. To sense light quantity and quality plants possess different types of photoreceptors, of which the red-/far-red light-absorbing phytochrome is the best characterized. The existence of a number of phytochrome proteins encoded by different genes (Pratt 1995; Quail 1994), which in tomato can have specific expression patterns (Hauser et al. 1995), adds to the complexity of phytochrome signal transduction. It is assumed that the different phytochrome proteins share the same linear tetrapyrrole chromophore (Terry et al. 1993). Whether the different phytochromes use the same or different signal transduction pathways is not yet clear.

The genetic basis of photomorphogenesis can be investigated by using mutants specific for the photoreceptor itself or for the transduction chain.

Such mutants can help in attributing physiological roles to specific components of the system. Mutants in the phytochrome A and B protein and chromophore mutants, which are deficient in all types of phytochrome, have been described for several species (Koornneef and Kendrick 1994). Two different classes of signal transduction mutants have been identified: mutants that apparently mimic the light signal, such as the *det* and *cop* mutants in *Arabidopsis* and mutants resembling the photoreceptor mutants in overall phenotype such as *hy5* in *Arabidopsis* (reviewed by McNellis and Deng 1995). By molecular characterization of mutants at the protein, mRNA and DNA level it is possible to link specific mutants with cloned genes. The localization of a mutant and a specific gene at similar positions on the genome is often an important indication that the mutation affects that particular gene. When sequence information of a mutant allele is not available, the map position of a locus defined by a mutant phenotype can be a starting point for cloning that particular locus (Tanksley et al. 1995). A problem, when comparing map positions of mutants on the classical map with positions of molecular markers on the molecular map, is that often different mapping populations and reference markers have been used for the construction of both type of maps. A further complication, *e.g.* in a species such as tomato, is that the classical maps (Tanksley et al. 1992) are often inaccurate. To solve the problem of the integration of classical and molecular maps, morphological markers should be mapped in relation to molecular markers and, in addition, the quality of the classical marker map has to be improved. The latter can be achieved by adding new data and using software that is able to integrate linkage data. In tomato (*Lycopersicon esculentum* Mill.) this integration of classical and molecular maps has already been done for the top part of chromosome 1 (Balint-Kurti et al. 1995), for chromosome 3 (Koornneef et al. 1993; Van der Biezen et al. 1994), and for chromosome 6 (Van Wordragen et al. 1996; Weide et al. 1993). In addition, miscellaneous markers are integrated in the standard molecular map (Tanksley et al. 1992).

In tomato the phytochrome gene family appears to consist of as many as 9 to 13 members (Hauser et al. 1995) of which 5 (designated *PhyA*,

*PhyB1*, *PhyB2*, *PhyE* and *PhyF*) have been characterized more thoroughly (Hauser et al. 1995; Pratt 1995). The position on the linkage map, however, is still unknown. This is also true of some of the recently discovered or even the well known photomorphogenic mutants in tomato.

Sharrock et al. (1988), using a RFLP for a phytochrome coding sequence from *Arabidopsis*, which they presumed was *PhyA*, mapped this locus to tomato chromosome 10. Van Tuinen et al. (1995a) described the isolation and characterization of two allelic phyA-deficient mutants ($fri^1$ and $fri^2$) in tomato and reported linkage with chromosome 10 morphological markers. The fact that the phyA-deficient mutants mapped to chromosome 10 suggests that Sharrock et al. (1988) indeed mapped the *PhyA* homologous gene.

The *tri* mutant of tomato, unlike the previously described phyB-deficient mutants of *Arabidopsis* (Reed et al. 1993), cucumber (López-Juez et al. 1992) and *Brassica rapa* (Devlin et al. 1992) still exhibits a strong end-of-day far-red response (Van Tuinen et al. 1995b). Tomato, like *Arabidopsis*, has two type B phytochromes. However, analysis revealed that this has arisen as a result of independent duplications of a *PhyB* progenitor gene in the *Solanaceae* and *Brassicaceae* (Pratt et al. 1995). Molecular analysis of mRNAs of the *tri*-mutant alleles points to the *Tri* locus being the gene encoding the apoprotein of phytochrome B1 (Kerckhoffs et al. 1996). Localization at the same position on the chromosome would give further evidence for the view that the *tri* mutants are defective in the *PhyB1* gene itself.

Chromophore mutants in tomato are represented by the *au* and *yg-2* mutations (Van Tuinen et al. 1996; Terry and Kendrick 1996). The position of the *au* mutant is firmly established on the classical as well as the RFLP map of chromosome 1 (Balint-Kurti et al. 1995). For *yg-2*, however, the suggestion that it might be on chromosome 12 (Kerr 1979c) has never been confirmed.

A type of transduction chain mutant that thus far has only been described in tomato is the *high pigment* (*hp*) mutant (Peters et al. 1989), which shows an exaggerated phytochrome response, and no difference in the phytochrome content compared to wild type has yet been found (Peters et al.

1992). The first *hp* gene was reported as early as 1917 (Reynard 1956). Soressi (1975) described a mutant that resembled *hp* phenotypically, but found that it was non-allelic to *hp* and designated it *hp-2*. Mochizuki and Kamimura (1986), however, observed an *hp* phenotype in the $F_1$ and a lack of segregation in $F_2$ populations derived from crosses between *hp* and *hp-2* and concluded that *hp-2* was allelic to *hp*.

The present report describes the mapping of 5 different phytochrome genes in tomato on the basis of their RFLP pattern using gene specific probes. In addition, we mapped the recently isolated *fri* and *tri* mutants. The map positions confirmed their relationship with the *PhyA* gene on chromosome 10 and *PhyB1* gene on chromosome 1, respectively. This allowed a better integration of the classical and molecular maps of chromosomes 1 and 10. A revised classical map was made for chromosome 10 by adding recent linkage data to all data on chromosome 10 available in the literature. Additional linkage data were obtained for *au* and *yg-2*, whose location on chromosome 12 was confirmed. The conflict in literature on the existence of a distinct *hp-2* locus was resolved and the gene was mapped on chromosome 1, using both morphological and RFLP markers.

## Results and discussion

### Mapping of the *tri* and *hp-2* loci, and the *PhyB1* gene on chromosome 1

Mochizuki and Kamimura (1986) stated that *hp-2* (Soressi 1975) was allelic to *hp*. Based on our allelism tests between *hp-2* and different *hp* mutants (A. van Tuinen unpublished data), we concluded that *hp-2* is not allelic to *hp*. Moreover, the tests revealed a second *hp-2* (*hp-2*$^j$) allele. We therefore propose to change the gene symbol *hp* into *hp-1*.

Since it was difficult to find the *au,hp-2* and *au,tri* double mutants we suspected both *hp-2* and *tri* to be located on chromosome 1. The *hp-2* and *tri* mutants were therefore crossed with lines carrying chromosome 1 morphological markers and linkage was detected (Table 1). Our data (Table 1) combined with those used by Balint-Kurti et al. (1995) were used to construct a revised classical map of the top part of chromosome 1 (Fig. 1). For the construction of the RFLP map individual plants of 4 BC populations were scored for *tri*, *hp-2* or wild-type phenotype and used for RFLP analysis. The BC data were combined with data from the $F_2$ population using JOINMAP (Stam 1993) to construct the RFLP map (Fig. 1). The absence of recombinants between *tri* and PHYB1 is consistent with the observation that mRNA analysis points to the *Tri* locus being the gene encoding the apoprotein of phytochrome B1 (Kerckhoffs et al. 1996). On the RFLP map the *tri* and *hp-2* genes map to the same position on the chromosome. This coincides with their similar position on the updated classical map (Fig. 1).

## A revised classical map of chromosome 10 and molecular mapping of the *PhyA* gene

We reported previously that the phyA-deficient *fri* mutants mapped to chromosome 10 (Van Tuinen et al. 1995a). The map position of *fri* was then calculated relative to the map position of *u* on the linkage map published by Tanksley et al. (1992). Linkage data presented for the *fri* mutants (Van Tuinen et al. 1995a) and those from literature (Burdick 1958; Butler 1955, 1956; Butler and Chang 1958; Chiscon 1960a; Clayberg 1962; Hansen et al. 1962; Kerr 1958, 1960, 1966, 1967, 1979a, 1982a, 1982b, 1982c; McArthur 1934; Mutschler 1984; Reeves et al. 1967; Rick 1955, 1956; Rick and Fobes 1977; Rick et al. 1968, 1974; Tigchelaar and Barman 1978; Tigchelaar et al. 1973) that could be verified (*e.g.* recalculated with the computer programme RECF2 (Koornneef and Stam 1992)) were combined to construct the revised classical map of chromosome 10 (Fig. 1), using the computer programme

**Table 1.** New linkage data on classical markers on chromosome 1

| Markers | Phase[a] | Genotype classes | | | | Total | $\chi^2_{ass}$ | Rec.[b] |
|---|---|---|---|---|---|---|---|---|
| | | *A.B.* | *aaB.* | *A.bb* | *aabb* | | | |
| *au - hp-2* | C | 342 | 34 | 26 | 76 | 478 | 193.8* | 14.1 ± 1.7 |
| *au - tri* | C | 259 | 21 | 26 | 62 | 368 | 152.4* | 14.1 ± 2.0 |
| *au - gib-2* | R | 254 | 98 | 116 | 12 | 480 | 18.1* | 32.0 ± 4.0 |
| *au - scf* | C | 233 | 35 | 68 | 13 | 349 | 0.5 | 45.7 ± 3.8 |
| *hp-2 - gib-2* | R | 256 | 96 | 120 | 6 | 478 | 28.0* | 23.9 ± 4.3 |
| *hp-2 - scf* | R | 136 | 38 | 32 | 6 | 212 | 0.7 | 46.7 ± 5.3 |
| *tri - scf* | R | 102 | 40 | 19 | 4 | 165 | 1.2 | 41.2 ± 6.4 |

* Significant at $p < 0.001$

[a] C: coupling phase; R: repulsion phase

[b] Rec. is recombinant percentage with SE calculated with RECF2

JOINMAP (Stam 1993). The *ten* locus could no longer be placed on the map because only reliable linkage data with one marker (Reeves et al. 1966) were available. For *sh* the indication of linkage to chromosome 10 (Lesley and Lesley 1971, 1980) was later confirmed by Kerr (1982c). The data on linkage for *sh* with *h* and *t* obtained by Kerr (1982c) could not be used, due to the lethality of the *XaXa* plants, leaving only an estimation of linkage with *Xa*. Although Andrásfalvy (1968) reported linkage with *h* for *ms-31*, the results of the trisomic analysis by Reeves (1972) were inconclusive. No further data for *ms-31* have been found, a position on chromosome 10 is therefore tentative. Rick et al. (1994) reported *Abg*, a gene for purple fruit derived from *Solanum lycopersicoides*, which co-segregated with the chromosome 10 RAPD marker Do3-1200. Further data are necessary to verify its position on chromosome 10. Semi-dominant chlorophyll mutants with seedling lethality of the homozygous dominant have been found in tomato and named *Xanthophyllic* (*Xa*). Three different *Xa* loci have been described: *Xa* (Young and McArthur 1947), *Xa-2* (Persson 1960; Anonymous 1963) and *Xa-3* (Gröber 1963). However, no reports of allelism tests were found in the literature. Due to the absence of linkage data that could be verified, *Xa-2* could not be placed on the new classical map (Fig. 1). The fact that *Xa* and *Xa-3* map to approximately the same position on the new classical map might indicate allelism. The position of *oli* on the map published by Tanksley et al. (1992) is based on Kerr's linkage summary (1977). However, Kerr did not include his earlier linkage data with *u* and *h* (Kerr 1967) or the linkage data with *h* presented by Hansen et al. (1962). The movement of *oli* from the long arm to the short arm can therefore be explained by the facts that it is unlinked to *ag* (Kerr 1967), the relative loose linkage with *t* and *l-2* (Kerr 1977) and the 'forgotten' stronger linkage data with *h* (Hansen et al. 1962; Kerr 1967).

The *PhyA* gene was mapped by RFLP analysis to the TG52 cluster on chromosome 10 (Fig. 1). The fact that the *fri* gene maps to a position on the classical map comparable to that of the *PhyA* gene on the RFLP map supports the hypothesis that the *fri* mutants are defective in the *PhyA* gene

itself. Preliminary results indicate that the DNA sequence of both *fri* alleles differs from the wild-type DNA sequence (G. Lazarova et al. 1996).

## Molecular mapping of the *PhyB2*, *PhyE* and *PhyF* genes

The DNA sequence homology of the *PhyB2* gene with the *PhyB1* gene is 84% for the 212-430 region (Pratt et al. 1995). The *PhyB2* gene does not map in the vicinity of the *PhyB1* gene on chromosome 1, but maps to chromosome 5 (Fig. 1). The *PhyE* and *PhyF* genes were located on chromosomes 2 and 7, respectively (Fig. 1).

## Mapping of the *yg-2* gene

The classical linkage map of chromosome 12 is poorly developed and contains only the markers *alb*, *fd* and *mua*, the position of the latter even indicated with a question mark (Tanksley et al. 1992). The reliability of the *Cf-11* position has also been questioned (P. Lindhout personal communication). Linkage of *yg-2* with *alb* and *fd* has been found (Kerr 1979c; Rick et al. 1968), but the locus was not placed on the map (Tanksley et al. 1992).

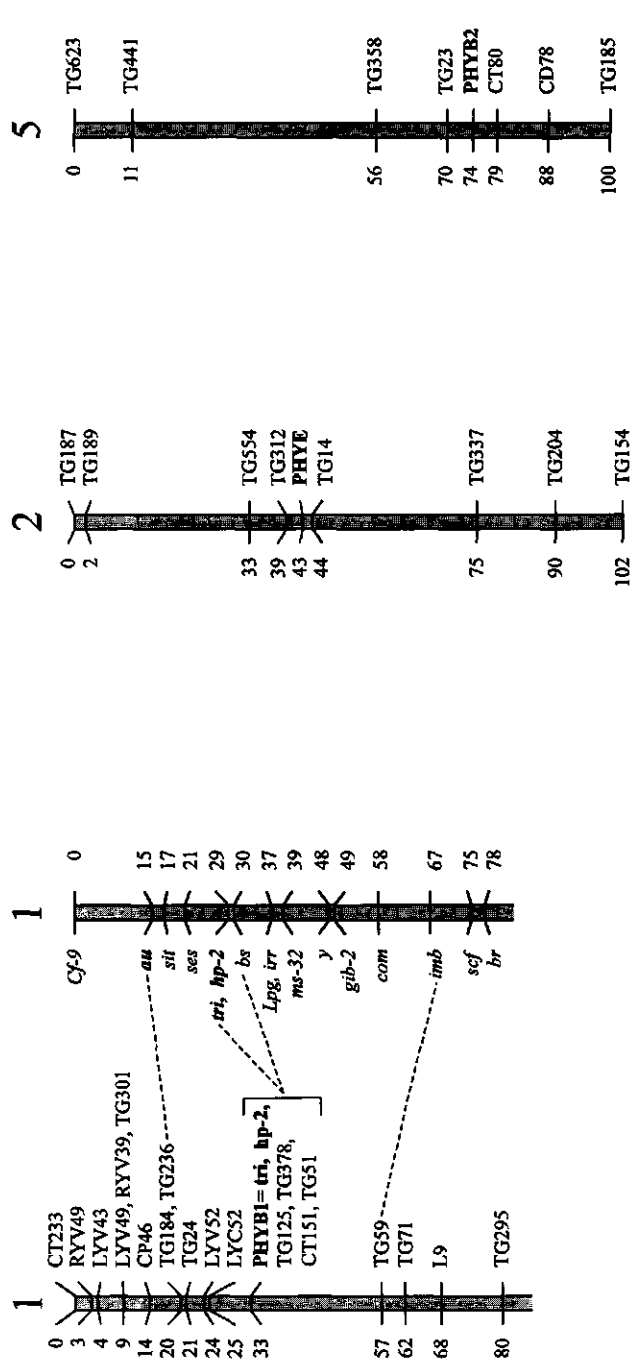
Linkage analysis of $F_2$ populations derived from crosses of the *yg-2* mutant with tester lines homozygous for several morphological markers of chromosome 12 revealed significant linkages with all markers used, thus confirming the observation of Kerr (1979c). Therefore all linkage data on chromosome 12 were collected and checked with the computer programme RECF2 (Koornneef and Stam 1992). After this check the literature data were pooled with our data and the recombination percentages given in Table 2 obtained. The revised classical map constructed with these data is given in Figure 1.

Based upon linkage data for *hp-1* and *yg-2* from two small $F_2$ populations in repulsion phase and very weak linkage of *hp-1* with *alb* and *fd*, Kerr

**Table 2.** Data on linkage between classical markers on chromosome 12

| Markers | Phase[a] | Genotype classes | | | | Total | $\chi^2_{ass}$ [b] | Rec.[c] |
|---|---|---|---|---|---|---|---|---|
| | | *A.B.* | *aaB.* | *A.bb* | *aabb* | | | |
| *alb - yg-2* | R | 462 | 219 | 191 | 8 | 880[d] | 63.6 | 20.0 ± 3.2 |
| *alb - fd* | C | 144 | 25 | 18 | 14 | 201 | 14.4 | 29.4 ± 3.9 |
| *alb - fd* | R | 1186 | 410 | 263 | 24 | 1883[e] | 41.2 | 34.3 ± 2.0 |
| *alb - mua* | C | 895 | 225 | 197 | 121 | 1438 | 43.7 | 37.8 ± 1.7 |
| *alb - mua* | R | 310 | 100 | 113 | 16 | 539[f] | 8.4 | 38.8 ± 3.6 |
| *yg-2 - fd* | R | 336 | 188 | 82 | 0 | 606[g] | 42.6 | 0.0 ± 4.1 |
| *yg-2 - mua* | C | 248 | 24 | 22 | 93 | 387 | 207.6 | 13.8 ± 2.2 |
| *yg-2 - mua* | R | 108 | 52 | 71 | 1 | 232 | 27.2 | 11.9 ± 6.5 |
| *fd - mua* | R | 384 | 108 | 236 | 0 | 728[f] | 60.8 | 0.0 ± 3.7 |

[a] C: coupling phase; R: repulsion phase
[b] All data are significant at 0.01 probability level
[c] Rec. is recombination percentage ± SE calculated with RECF2
[d] data from Rick et al. (1968) and Kerr (1979b) are included
[e] literature data only (Rick et al. 1968)
[f] data with recombinant percentage < 50 from Zobel et al. (1969) are included
[g] data from Rick et al. (1968) are included


1
0 CT233
3 RYV49
4 LYV43
9 LYV49, RYV39, TG301
14 CP46
20 TG184, TG236
21 TG24
24 LYV52
25 LYC52
33 PHYB1= tri, hp-2,
TG125, TG378,
CT151, TG51
57 TG59
62 TG71
68 L9
80 TG295
1
0 Cf-9
15 au
17 sit
21 ses
29 tri, hp-2
30 bs
37 Lpg, irr
39 ms-32
48 y
49 gib-2
58 com
67 imb
75 scf
78 br
2
0 TG187
2 TG189
33 TG554
39 TG312
43 PHYE
44 TG14
75 TG337
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5
0 TG623
11 TG441
56 TG358
70 TG23
74 PHYB2
79 CT80
88 CD78
100 TG185

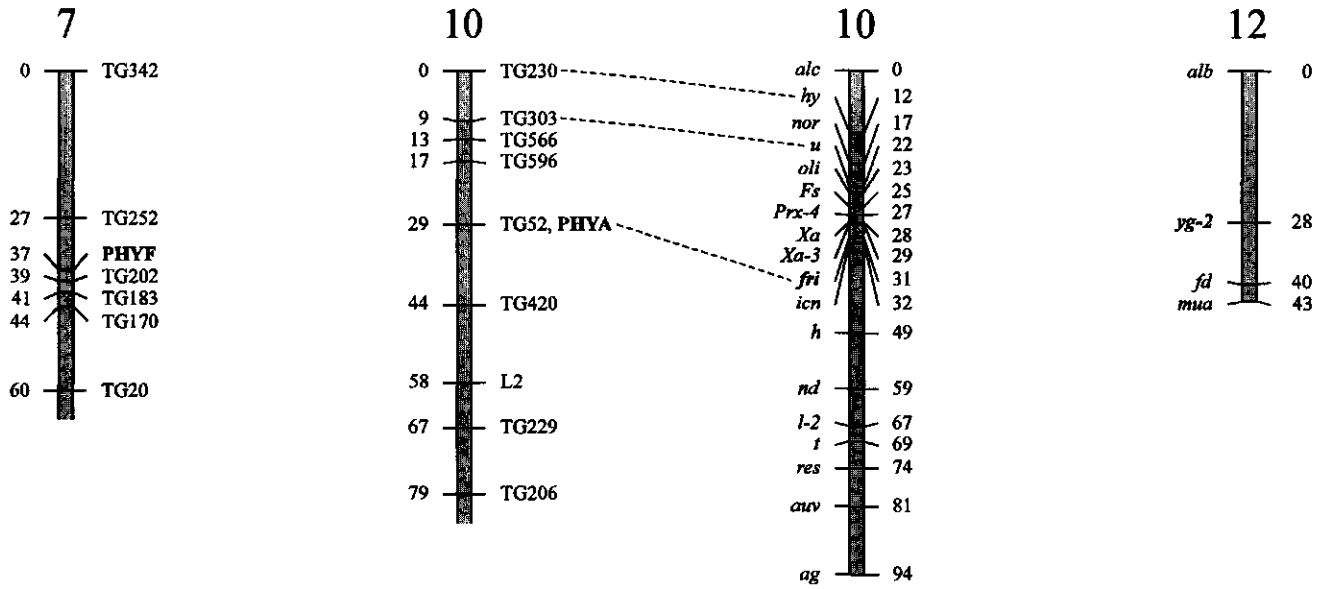


**Figure 1.** RFLP (markers to right) and classical (markers to left) maps of tomato chromosomes showing the map positions of the phytochrome genes and photomorphogenic mutants. Recombination distances for the RFLP maps of chromosomes 2, 5, 7 and 10 were based upon the analysis of 84 *L. esculentum* x *L. pennellii* $F_2$ plants (Odinot et al. 1992). For the RFLP map of chromosome 1 the $F_2$ data were combined with data on four *L. esculentum* x *L. pennellii* BC populations (see Materials and methods). The map orientations are according to maps published by Balint-Kurti et al. (1995; chromosome 1 classical map), Bonnema et al. (1996; chromosome 1 RFLP map), Frary et al. (1996; chromosome 7) and Tanksley et al. (1992; chromosomes 2, 5, 10 and 12). Dotted lines represent the integration between the RFLP and classical maps of chromosomes 1 (Balint-Kurti et al. 1995; this article ) and 10 (Tanksley et al. 1992; this article).

(1979b) concluded that *hp-1* also resided on chromosome 12. However, we found no significant linkage between *hp-1* and the chromosome 12 markers *alb* and *mua*. Moreover, when *hp-1* plants, out of an $F_2$ derived from a cross between *hp-1* and *yg-2*, were selected and allowed to self, 23 of the 30 $F_3$ lines obtained segregated *yg-2* plants. This does not significantly deviate from the expected 2 : 1 ($\chi^2$ = 1,35; $p > 0.05$) ratio of segregating to non segregating lines, giving no indication for linkage between *hp-1* and *yg-2* and indicating that *hp-1* is not on chromosome 12.

## Concluding remarks

Several phytochrome genes have been mapped in different species (Hauge et al. 1993; Paterson et al. 1995). In tomato, the phytochrome genes mapped so far are not clustered, but distributed over different chromosomes of the genome. This is in contrast to the cereals sorghum and rice, where the *PhyA*, *PhyB* and *PhyC* genes map to the same chromosome (A. Paterson, L.H. Pratt and M.-M. Cordonnier-Pratt personal communications), but comparable to *Arabidopsis* (Hauge et al. 1993).

The *au* and *yg-2* genes are most likely orthologues of the *Arabidopsis hy1* and *hy2* genes (Parks and Quail 1991). If cloning in *Arabidopsis* is successful, the *Arabidopsis* probes might be used in tomato to confirm co-localization.

The mapping of the *hp-2* and *tri* genes to the same cluster on chromosome 1 does not necessarily mean that the genes are related at the DNA level. The localization of *hp-2* and *tri* in a large cluster of markers might indicate suppression of recombination, which implies that the physical distances between the various markers might be considerable. In tomato such clusters of loci appear to be located near the centromeres (Tanksley et al. 1992). Recently, repeat sequences, which almost exclusively co-localize with centromeres, have been reported. One of these sequences was mapped to a position near TG51 on chromosome 1 (Broun and Tanksley 1996). The map

position of *hp-2* and *tri* in a region of low recombination will make map-based cloning difficult.

Mutants for the *PhyB2*, *PhyE* and *PhyF* genes (Hauser et al. 1995; Pratt 1995) have not yet been found. This might be due to the relatively small or specific effects of some members of the phytochrome gene family. Screening of the $M_2$ population derived from $\gamma$ radiation of *tri,fri* double-mutant seed has produced possible candidates for phytochrome deficiency at these loci (Koornneef et al. 1996).

## Materials and methods

### Plant materials

*Mutants*

The *fri* and *tri* mutants used have been described before by Van Tuinen et al. (1995a, 1995b), and the *au* and *yg-2* mutants by Koornneef et al. (1985), Van Tuinen et al. (1996) and references therein. A mutant with dark-green immature fruits, similar to the extreme *hp* allele, $hp^w$ (WB3) (Peters et al. 1989) was obtained from Drs David and Jonathan Jones (Sainsbury laboratory, Norwich, UK), who found this mutant in the progeny of a T-DNA transformed plant (cv. Moneymaker). This mutant phenotype did not co-segregate with the T-DNA. Allelism tests revealed that the mutant is allelic to the *hp-2* mutant (Soressi 1975) received from Dr. Soressi. We propose the gene symbol $hp\text{-}2^j$ for this new *hp-2* allele.

Genotypes, homozygous for several morphological markers were obtained from the Tomato Genetic Resource Center (Davis, CA, USA) or have been constructed in Wageningen (Koornneef et al. 1990).

*Mapping populations*

For the molecular mapping of the *tri* and *hp-2* mutants several backcross (BC) populations have been used. A *L. esculentum* line carrying the $tri^1$ mutant allele was crossed with *L. pennellii* (LA716). An $F_1$ plant was used as a male parent in a backcross to the *esculentum* line carrying the $tri^1$ mutant allele. This generated a $BC_1$ population of 28 plants. For *hp-2* the procedure was followed as outlined above. In the *pennellii* background, however, it was impossible to score the *hp* phenotype (Peters et al. 1989) unambiguously, since both *hp* seedling phenotype and fruit colour were modified by *L. pennellii* genes and intermediate phenotypes were found. From this $BC_1$ population DNA was isolated from plants with clear phenotypes only, generating a population of 20 individuals. Two $BC_1$ plants with clear wild-type fruit colour were selected and backcrossed again, this time to a

line carrying the more extreme *hp-2$^j$* allele described above. This generated 2 $BC_2$ populations of 42 and 49 individuals, respectively, which segregated in a ratio of 1 : 1 for wild-type to *hp* fruit colour, indicating the absence of modifying loci. The *tri* and *hp-2* phenotypes were scored at the seedling level after a 7-day period of continuous red light (Philips TL40/103339 filtered through one layer of primary red filter (Lee, Flashlight Sales BV, Utrecht, The Netherlands; 3 µmol $m^{-2}$ $s^{-1}$). The *hp* phenotype was confirmed at the adult plant stage by fruit colour.

To locate the various phytochrome genes an $F_2$ mapping population (84 plants), derived from a cross between *L. esculentum* cv. Allround and *L. pennellii* (LA716) (Odinot et al. 1992), was used. DNA of these plants was kindly provided by Dr. P. Lindhout (Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen, The Netherlands).

For the mapping of *yg-2*, the *yg-2* mutant was crossed with a line (LA1177) carrying the chromosome 12 markers *alb* (Khush and Rick 1966) and *mua* (Zobel et al. 1969). Linkage analysis performed in the $F_2$ population produced 1 *yg-2,mua* recombinant, which did not segregate *alb* in the $F_3$. To obtain more linkage data the *yg-2,mua* line was crossed to lines carrying the *fd* mutation (Rick et al. 1967; LA873) or the *alb* and *fd* mutations (LA1111).

## DNA methodology

Plant DNA was isolated from leaves as described by Van der Beek et al. (1992), digested with the appropriate restriction enzymes, separated by electrophoresis on 1% (w/v) agarose gels, denatured and blotted onto Genescreen Plus (NEN Research Products, Boston, MA) hybridization membranes. Probes were labelled with $^{32}$P-dATP using the "random primer" method according to Feinberg and Vogelstein (1983). Hybridization of Southern blots was performed as stated by Klein-Lankhorst et al. (1991).

## Probes and hybridization conditions

For the molecular mapping of the phytochrome genes PHY fragments ($PHYA_{212/320}$, $PHYB1_{212/430}$, $PHYB2_{212/430}$, $PHYE_{212/320}$ and $PHYF_{212/320}$; Hauser et al. 1995) were cut out of their bluescript vector and segregated from vector DNA on a 1% agarosegel. The tomato DNA was isolated from the agarose using the GENECLEAN (Bio 101 Inc., La Jolla, CA) procedure according to the manufacturers instructions. The cleaned DNA was then used for the preparation of radioactive labelled probes.

The phytochrome genes were mapped using tomato genomic (TG-) clones, with known positions, developed by Tanksley et al. (1992). After PCR of the inserts using M13 primers the PCR product was used as a probe either directly or after cleaning by the freeze-squeeze method (Ogden and Adams 1987).

## Linkage analysis and map construction

In order to identify RFLPs suitable for mapping the phytochrome genes, DNA from *L. esculentum* cv. Moneymaker and *L. pennellii* (LA716) was digested with 14 restriction enzymes. Southern-blot analysis, using the radioactive labelled PHYA, PHYB1, PHYB2, PHYE and PHYF probes, revealed polymorphisms for TaqI, HindIII, HindIII, HincII and HaeIII, respectively. Subsequently, the probes were hybridized to Southern blots containing DNA, digested with the appropriate restriction enzyme, of the $F_2$ population described above (plant material). This $F_2$ population, characterized with several RFLP markers common to the map constructed by Tanksley et al. (1992), has been used previously for mapping studies (Arens et al. 1995; Bonnema et al. 1996; Liharska et al. 1994). We added our data on the phytochrome genes and screened the population with additional RFLP markers. These were TG51, TG125 for chromosome 1; TG14, TG204, TG312, TG554 for chromosome 2; CD78, CT80, TG623 for chromosome 5; TG183, TG202 for chromosome 7 and TG303, TG566, TG596 for chromosome 10 (Tanksley et al. 1992). The BC populations were scored for the segregation of either *tri* or *hp-2* and the RFLP segregation for PHYB1, LYC52 (Bonnema et al. 1996) and chromosome 1 markers TG24, TG51, TG125, TG378 and CT151 (Tanksley et al. 1992).

Segregation data were analyzed with the computer programme JOINMAP (Stam 1993). Graphical depiction of the maps was accomplished by the programme DRAWMAP (Van Ooijen 1994).

**Acknowledgements** This research was supported by a grant to A.v.T. from the Foundation for Life Sciences (SLW) formerly the Foundation for Biological Research (BION) which is subsidized by the Netherlands Organization for Scientific Research (NWO). We thank: D. and J. Jones for seeds of the *hp-2$^j$* mutant; P. Lindhout for provision of the $F_2$ mapping population; G. Bonnema for discussions regarding the RFLP work and R.E. and C.I. Kendrick for critically reading of the manuscript.

# Chapter 6

# General discussion

# General discussion

Light is of the utmost importance for plant growth. Plants need light as a source of energy and information. After germination, a seedling changes its growth habit upon emergence through the soil surface from a dark-adapted (etiolated) seedling to a light-adapted photosynthetic plant. This so called "de-etiolation" is an example of photomorphogenesis, which comprises all the responses of a plant to the information content of the light environment. To sense light quantity and quality plants possess different groups of photoreceptors: those that absorb in the UV-B, the UV-A, the blue light (B)-absorbing cryptochromes, and the red light (R)/far-red light (FR)-absorbing phytochromes. Mutants deficient in one of these photoreceptors can be used to reveal the contribution of the photoreceptor concerned and its interaction in the regulation of photomorphogenic responses. How important this genetic approach is has been emphasized by Smith (1995), who stated that the most important, and potentially the most powerful, advance in phytochrome research has been the selection of a range of photomorphogenic mutants.

Hypocotyl elongation, as a part of the de-etiolation process, is controlled by different photoreceptors and has been used as a screening tool for photomorphogenic mutants. Most photomorphogenic mutants found display an elongated (*e.g.* a long-hypocotyl) phenotype, either in white light (WL) and/or under broad-band light sources. However, not all long-hypocotyl mutants should be considered photomorphogenic mutants. A long-hypocotyl phenotype is also exhibited by the *la,cry*$^{s}$ mutant of pea (Reid and

Ross 1993), the *sln* mutant of barley (Lanahan and Ho 1988), the *spy* mutant of *Arabidopsis* (Jacobsen and Olszewski 1993) and the *pro* mutant of tomato (Jones 1987; A. van Tuinen et al. manuscript in prep), which are constitutive gibberellin (GA) response mutants. These mutants are epistatic to GA deficiency, either chemically or genetically imposed. This epistatic relationship is not shown by photoreceptor mutants. Recently, a third type of long hypocotyl mutant has been found in *Arabidopsis* with the isolation and characterization of the *elg* mutant. The ELG gene product appears to mediate elongation growth through a transduction pathway that is separate from the photoreceptor- or GA mediated signal transduction chains. The resemblance of the *elg* mutant phenotype to that of auxin overproducers could indicate a relationship with this hormone (Halliday et al. 1996).

## Photoreceptor mutants

At the start of this study the only long hypocotyl, photomorphogenic, mutants available in tomato were the *au* and *yg-2* mutants. Based upon the absence of functional phytochrome A (phyA) in dark-grown seedlings (Koornneef et al. 1985; Parks et al. 1987) and the presence of an end-of-day FR (EODFR) response (Adamse et al. 1988), which is commonly accepted to be mainly regulated by phyB (Adamse et al. 1987; Devlin et al. 1992; López-Juez et al. 1992; Reed et al. 1993), the *au* mutant was thought to be specifically phyA deficient. However, Sharrock mapped a *PHYA*-coding sequence from *Arabidopsis* to chromosome 10, whereas *au* and *yg-2* map to chromosomes 1 (Balint-Kurti et al. 1995) and 12 (Kerr 1979c), respectively. The isolation of the phyA-deficient *fri* mutants of tomato (Chapter 2), which resemble the phyA mutants of *Arabidopsis* (Nagatani et al. 1993; Parks and Quail 1993; Whitelam et al. 1993) and have a phenotype clearly distinguishable from that of the *au* and *yg-2* mutants, justified the doubt which had arisen about the nature of the *au* and *yg-2* mutations. In addition, the *fri* locus mapped to chromosome 10 at a position similar to that of the phyA

gene on the RFLP map (Chapter 5). Combination, by crossing the *au* and *yg-2* mutants with a transgenic tomato line that overexpresses the oat phytochrome A3 gene essentially failed to restore the wild-type (WT) phenotype under white fluorescent light. Although, immunochemically oat PHYA-3 protein is detectable in both *au,PhyA-3* and *yg-2,PhyA-3* 'double' mutants, spectrophotometrical analysis revealed that holophytochrome was undetectable in both (Chapter 4). The results were compatible with both mutants being disturbed in phytochrome chromophore biosynthesis. Recently, Terry and Kendrick (1996) defined the biochemical lesions of *au* and *yg-2* in two different steps of the chromophore biosynthesis pathway. These mutants resemble therefore the *hy1* and *hy2* mutants of *Arabidopsis* (Parks and Quail 1991) and the *pcd1* mutant of pea (Weller et al. 1996). The reduced level of PHYA protein in *au* and *yg-2* (Parks et al. 1987; Sharma et al. 1993; Van Tuinen et al. 1996) compared to the normal levels in the *Arabidopsis* and pea mutants, indicates that phytochrome without a chromophore is apparently unstable in tomato.

Four recessive mutants were selected, that resembled the phyB-deficient mutants of *Arabidopsis* (Reed et al. 1993), cucumber (López-Juez et al. 1992), *Brassica rapa* (Devlin et al. 1992) and pea (Weller et al. 1995) in exhibiting an elongated phenotype in continuous R. Kerckhoffs et al. (1996) found that the *tri* mutant alleles differed from WT quantitatively or qualitatively (truncated products) in PHYB1 protein or PHYB1 mRNA. The mapping of the *PhyB1* gene and *tri* mutant gene at a similar position on chromosome 1 (Chapter 5) confirms that the two loci are identical. In contrast to the mutants found in other species, the 4 allelic *tri* mutants were only insensitive to R during the first 2 days upon transition from darkness to R and responded to EODFR treatment and supplementary FR. Although, slightly elongated in WL, the *tri* mutants are much less extreme than phyB-deficient mutants of other species in WL. In tomato, the phytochrome gene family comprises two phyB genes (Hauser et al. 1995, Pratt et al. 1995). The absence of functional phyB1 in the *tri* mutants indicates that the shade avoidance process, thought to be regulated by phyB exclusively seems, at

least in tomato, to involve more than one light-stable type of phytochrome. It would be interesting to know if this other phytochrome involved is phyB2. However, a closer examination in a more sensitive background, showed that the EODFR response on flowering is still exhibited by the phyB-deficient mutant of *Arabidopsis* (Devlin et al. 1996; Halliday et al. 1994) and other features of the shade avoidance syndrome are also unaffected by the absence of phyB (Robson et al. 1993). This led to the conclusion that also in *Arabidopsis* additional stable-type phytochromes are involved. The *Arabidopsis* phyD gene is now regarded as a phyB subfamily member, based on its sequence homology (Clack et al. 1994). Recently, a phyD deficient Arabidopsis has been identified and the analysis of this genotype and double mutants with phyB indicate that phyD regulates several of the same R responses as phyB (Sharrock 1996). Therefore, the difference between tomato and other species seems not to be the presence of two rather than one phyB gene, but to lie in the relative contribution of phyB1 compared to phyB in other species, suggested by the less extreme phenotype of the *tri* mutants.

In our screens other clearly R or B insensitive mutants were not found, it seems therefore unlikely that a mutation in one of the other phytochromes or B photoreceptor is extreme. Since the expression level of the various phytochrome genes differs quantitatively between genes, per gene throughout the plant life cycle and in spatial distribution (Hauser et al. 1995; Pratt 1995; Pratt et al. 1995), deficiencies in other phytochromes might cause only subtle effects or ones not visible at the seedling stage, which we used as a screen. Other selection conditions might be necessary to find such mutants. The *tri* mutant, for instance, has a more pronounced, *i.e.* elongated, phenotype in low-irradiance WL as compared to a light-saturating environment. However, some mutants with subtle phenotypes have been selected. The Z3-78 mutant, for instance, is slightly longer in B, R and WL and has a lower anthocyanin content. However, as a double mutant in combination with the *tri* or *au* mutants, the phenotype in WL is more extreme, exhibiting hypocotyl elongation to a greater extent than that of the single mutants and complete

absence of anthocyanins. This synergistic effect is most pronounced by the Z3-78,*au* double mutant, where the seedlings have an almost etiolated phenotype in WL. The extreme elongated phenotype persists during further development, although some normalisation in chlorophyll occurs at a later stage. Both the Z3-78,*tri* and Z3-78,*au* double mutants lack an EODFR response. The B2 mutant, although less inhibited by B and R than the Z3-78 mutant, also exhibits an additive effect on hypocotyl elongation, when combined with the *au* mutant. The 1-38BBL mutant is also slightly taller compared to WT in B and R. Preliminary data indicate that the response to EODFR is less than that of the WT. The synergistic effects of these subtle mutants in different double-mutant backgrounds, indicates that their gene products are involved in processes for which phyA and phyB1 can compensate, *i.e.* redundancy of the phytochrome system. However, biochemical evidence that these mutants are affected in one of the other phytochromes is lacking at this moment. Analysis of these mutants with tomato phytochrome gene-specific probes and type-specific antibodies currently under production are eagerly awaited.

The *fri,tri* double mutant resembles the *tri* mutant in WL. This indicates that phyA is not the phytochrome that compensates for the loss of phyB1 in WL, confirming that phyA and phyB1 have distinct physiological roles. With the *fri,tri* double mutant as starting material for mutagenesis it was predicted that mutants of other phytochromes or the B receptor(s) could be identified, since their loss could not be compensated for by phyB1. At least eight independently induced mutants, which are extremely tall in WL, have been isolated at this moment (Koornneef et al. 1996). It has to be awaited what the phenotypes of the single mutants will be in the absence of phyA and phyB1 deficiency. The availability of cloned tomato phytochrome genes (Pratt 1995) and the tomato homologue of the *Arabidopsis* B photoreceptor gene *HY4* (J. Giuliano personal communication), should allow an efficient molecular characterization of these mutants, when they are affected in one of these cloned genes. Since these mutations were induced by γ-irradiation, which often leads to deletions, such mutations in photoreceptor

genes might be detected by Southern and Northern analysis. The mapping of these mutations could also indicate their identity with cloned genes, since the positions of three phytochrome genes, for which no mutants are characterized as such (*PhyB2*, *E* and *F*), are known (Chapter 5). However, the dependence of the phenotype on other mutants will complicate linkage analysis.

## Regulatory mutants

The *hp* mutant is characterized by dark-green foliage and immature fruit colour and high anthocyanin content (Kerr 1965; Von Wettstein-Knowles 1968). The mature fruits exhibit a marked increase in firmness of the flesh and due to higher lycopene and carotene content are deep red (Thompson 1961). Commercial plant breeders have been interested in introducing these characteristics into their cultivars, however, without the pleiotropic effects, such as a lower seedling growth rate (Thompson 1961). The phenotype of the *hp* mutant results from exaggerated phytochrome responses, although no difference in the phytochrome content compared to WT has been found (Peters et al. 1992). The *hp* mutant no longer requires co-action of the B photoreceptor and phytochrome alone can achieve normal photomorphogenesis (Oëlmüller and Kendrick 1991). The HP gene product is proposed to inhibit the amplification of the phytochrome response, which can be overcome by B (Peters et al. 1992). Due to conflicting data on allelism tests with a *hp*-like mutant isolated by Soressi (1975; Mochizuki and Kamimura 1986), it was not clear whether there were two *hp* genes or one *hp* locus with two alleles. Allelism tests with our available *hp* mutants revealed that the *hp* mutant of Soressi (1975) was not allelic to the *hp* mutant of Kerr (1965), as already stated by Soressi (1975), who designated his mutant *hp-2*. Two additional *hp-1* alleles were isolated, exhibiting short-hypocotyl phenotypes in our screens for photomorphogenic mutants. Another *hp*-like mutant, that was sent to us by David Jones (Sainsbury Laboratory, Norwich, UK), turned out to be a new and stronger allele of *hp-2*. The *hp-2* gene mapped to

chromosome 1 (Chapter 5), whereas the *hp-1* gene maps to the top of chromosome 2 (J. Giovannoni personal communication). Unfortunately both the *hp-1* and *hp-2* genes are located in parts of the chromosomes that are not favourable for map based cloning, because of repressed recombination near the centromere region of chromosomes (Broun and Tanksley 1996). Recently, the isolation of *her*, a *hp*-type mutant in *Arabidopsis* has been reported (Ichikawa et al. 1996). If this really is the *Arabidopsis* homologue of the tomato *hp* genes, cloning of this gene might be easier than cloning of the tomato *hp* genes. The *hp-1* mutant in combination with the *fri* and *tri* mutants has been a very useful tool to study the influence of phyA and phyB1 on anthocyanin production in tomato. Threshold-box experiments, using R, revealed that both phyA and phyB1 influence anthocyanin biosynthesis, but do so via different modes; phyA controls the low fluence response rate and phyB1 the high irradiance response. It has been shown that the *hp-1* mutation amplifies the remaining anthocyanin response in both *fri* and *tri* mutants. This indicates that *hp-1* is associated with the amplification step downstream of specific phyA and phyB1 reactions at a later common step in the anthocyanin signal transduction chain (Kerckhoffs et al. in press).

Two other mutants which influence anthocyanin levels in tomato are the *atv* and *Ip* mutants. The *atv* mutant has elevated anthocyanin levels, but does not show the dark-green fruit colour of the *hp-1* and *hp-2* mutants. Compared to WT anthocyanin levels are elevated and hypocotyl inhibition is only more pronounced under R. The *Ip* mutant has slightly darker green unripe fruit colour in WL, whereas effects on anthocyanin level and hypocotyl inhibition are enhanced by B, but not R (Kerckhoffs et al. in press). The *Ip* phenotype is difficult to recognize and therefore the genetics of the mutant is unclear. However, the fact that the *Ip* and *atv* mutants originate, respectively, from the wild species *Lycopersicon chmielewski* (Rick 1974) and *Lycopersicon cheesmannii* (Rick et al. 1968), and have been crossed into *L. esculentum* is an advantage if the *Ip* and *atv* genes are to be cloned, because introgressed regions in near isogenic lines are an attractive starting material for the identification of molecular markers, which

subsequently can serve as starting points for map based cloning.

Although, the $M_2$ populations that generated the *fri* mutants, a *tri* allele and two *hp-1* alleles, have been screened in the dark to find the tomato equivalents of *cop* and *det* mutants, no such mutants were found, whereas in *Arabidopsis* they are numerous (McNellis and Deng 1995). Recently, Tsuge et al. (1996) reported that the *COP1* homologue of tomato has been cloned.

The research described in this thesis has produced additional photoreceptor mutants of tomato, of which especially the *tri* mutant is intriguing, because of the difference from other phyB-deficient mutants. More mutants have been isolated and await further characterization. These mutants can be used to dissect the roles of individual components of the photomorphogenic system in this important crop and model plant.

# References

**Adamse P, Jaspers PAPM, Kendrick RE, Koornneef M** (1987) Photomorphogenetic responses of a long-hypocotyl mutant of *Cucumis sativa* L.. *J Plant Physiol* **127:** 481-491

**Adamse P, Jaspers PAPM, Bakker JA, Wesselius JC, Heeringa GH, Kendrick RE, Koornneef M** (1988) Photophysiology of a tomato mutant deficient in labile phytochrome. *J Plant Physiol* **133:** 436-440

**Adamse P, Peters JL, Jaspers PAPM, Van Tuinen A, Koornneef M, Kendrick RE** (1989) Photocontrol of anthocyanin synthesis in tomato seedlings: a genetic approach. *Photochem Photobiol* **50:** 107-111

**Ahmad M, Cashmore AR** (1993) *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature* **366:** 162-166

**Ahmad M, Cashmore AR** (1996) Seeing blue: the discovery of cryptochrome. *Plant Mol Biol* **30:** 851-861

**Andrásfalvy A** (1968) Linkage studies with *male-sterile* mutants. *Tomato Genet Coop Rep* **18:** 7

**Ang LH, Deng X-W** (1994) Regulatory hierarchy of photomorphogenic loci: Allele-specific and light-dependent interaction between the *HY5* and *COP1* loci. *Plant Cell* **4:** 801-811

**Anonymous** (1963) Linkage summary. *Tomato Genet Coop Rep* **13:** 3-4

**Anonymous** (1973) Report of the gene list committee. *Tomato Genet Coop Rep* **23:** 3-8

**Arens P, Odinot P, Van Heusden AW, Lindhout P, Vosman B** (1995) GATA- and GACA-repeats are not evenly distributed throughout the tomato genome. *Genome* **38:** 84-90

**Balint-Kurti PJ, Jones DA, Jones JDG** (1995) Integration of the classical and RFLP linkage maps of the short arm of tomato chromosome 1. *Theor Appl Genet* **90:** 17-26

**Becker TW, Foyer C, Caboche M** (1992) Light-regulated expression of the nitrate-reductase and nitrite-reductase genes in tomato and in the phytochrome-deficient *aurea* mutant of tomato. *Planta* **188:** 39-47

**Bonnema G, Hontelez J, Verkerk R, Zhang YQ, Van Daelen R, Van Kammen A, Zabel P** (1996) An improved method of partially digesting plant megabase DNA suitable for YAC cloning: application to the construction of a 5.5 genome equivalent YAC library of tomato. *Plant J* **9:** 125-133

**Boylan MT, Quail PH** (1989) Oat phytochrome is biologically active in transgenic tomatoes. *Plant Cell* **1:** 765-773

**Boylan MT, Quail PH** (1991) Phytochrome A overexpression inhibits hypocotyl elongation in transgenic *Arabidopsis*. *Proc Natl Acad Sci USA* **88:** 10806-10810

**Bowler C, Chua N-H** (1994) Emerging themes of plant signal transduction. *Plant Cell* **6:** 1529-1541

**Bowler C, Yamagata H, Neuhaus G, Chua N-H** (1994) Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. *Genes Dev* **8:** 2188-2202

*References*


**Broun P, Tanksley SD** (1996) Characterization and genetic mapping of simple repeat sequences in the tomato genome. *Mol Gen Genet* **250:** 39-49

**Burdick AB** (1958) New mutants. *Tomato Genet Coop Rep* **8:** 9-11

**Burdick AB** (1960) New gene symbols. *Tomato Genet Coop Rep* **10:** 8-9

**Burdick AB** (1961) New gene symbols and names. *Tomato Genet Coop Rep* **11:** 6

**Butler L** (1955) Dihybrid segregation for genes whose linkage relationship is unknown. *Tomato Genet Coop Rep* **5:** 10-13

**Butler L** (1956) Dihybrid $F_2$ segregations for genes in linkage groups VI & VII. *Tomato Genet Coop Rep* **6:** 11-12

**Butler L, Chang LO** (1958) Genetics and physiology of the *xanthophyllous* mutant of the tomato. *Can J Botany* **36:** 251-267

**Cherry JR, Hershey HP, Vierstra RD** (1991) Characterization of tobacco expressing functional oat phytochrome. *Plant Physiol* **96:** 775-785

**Childs KL, Cordonnier-Pratt M-M, Morgan PW** (1992) Genetic regulation of development in *Sorghum bicolor* VII. $ma_3^R$ Flowering mutant lacks a phytochrome that predominates in green tissue. *Plant Physiol* **99:** 765-770

**Chiscon JA** (1960a) Linkage of *nd*, *ag*, and *h*. *Tomato Genet Coop Rep* **10:** 11

**Chiscon JA** (1960b) Seedling identification. *Tomato Genet Coop Rep* **10:** 12

**Chiscon JA** (1961) A comparison of the content of chlorophylls *a* and *b* in some chlorophyll mutants. *Tomato Genet Coop Rep* **11:** 8-9

**Chory J** (1993) Out of darkness: Mutants reveal pathways controlling light-regulated development in plants. *Trends Genet* **9:** 167-172

**Chory J, Peto C, Feinbaum R, Pratt L, Ausubel F** (1989) *Arabidopsis thaliana* mutant that develops as a light-grown plant in the absence of light. *Cell* **58:** 991-999

**Chory J, Nagpal P, Peto CA** (1991) Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in *Arabidopsis*. *Plant Cell* **3:** 445-459

**Clack T, Mathews S, Sharrock RA** (1994) The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: The sequences and expression of *PHYD* and *PHYE*. *Plant Mol Biol* **25:** 413-427

**Clayberg CD** (1962) Inheritance and linkage of *fruit stripe*, *Fs*. *Tomato Genet Coop Rep* **12:** 22-23

**Darby LA, Ritchie DB, Taylor IB** (1978) Isogenic lines of the tomato 'Ailsa Craig'. *Annual Report Glasshouse Crops Research Institute* **1977:** 168-184

**Dehesh K, Franci C, Parks BM, Seeley KA, Short TW, Tepperman JM, Quail PH** (1993) *Arabidopsis HY8* locus encodes phytochrome A. *Plant Cell* **5:** 1081-1088

**Deng X-W, Quail PH** (1992) Genetic and phenotypic characterization of *cop1* mutants of *Arabidopsis thaliana*. *Plant J* **2:** 83-95

**Devlin PF, Rood SB, Somers DE, Quail PH, Whitelam GC** (1992) Photophysiology of the elongated internode (*ein*) mutant of *Brassica rapa*. *Plant Physiol* **100:** 1442-1447

**Devlin PF, Halliday KJ, Harberd NP, Whitelam GC** (1996) Novel phytochromes control flowering and internode elongation reponses in *Arabidopsis phyA phyB* mutants. In *Book of abstracts - 7th International Conference on* Arabidopsis *Research*, Norwich

**Feinberg AP, Vogelstein B** (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132:** 6-13

**Folch J, Lees M, Stanley GHS** (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226:** 496-509

**Foster KR, Miller FR, Childs KL, Morgan PW** (1994) Genetic regulation of development in *Sorghum bicolor* VIII. Shoot growth, tillering, flowering, gibberellin biosynthesis, and phytochrome levels are differentially affected by dosage of the $ma_3^R$ allele. *Plant Physiol* **105:** 941-948

**Frary A, Presting GG, Tanksley SD** (1996) Molecular mapping of the centromeres of tomato chromosomes 7 and 9. *Mol Gen Genet* **250:** 295-304

**Georghiou K, Kendrick RE** (1991) The germination characteristics of phytochrome-deficient *aurea* mutant tomato seeds. *Physiol Plant* **82:** 127-133

**Gröber K** (1963) Genetical and physiological behaviour of a dominant chlorophyll mutant of tomato. *Tomato Genet Coop Rep* **13:** 48

**Halliday KJ, Koornneef M, Whitelam GC** (1994) Phytochrome B and at least one other phytochrome mediate the accelerated flowering response of *Arabidopsis thaliana* L. to low red/far-red ratio. *Plant Physiol* **104:** 1311-1315

**Halliday KJ, Devlin PF, Whitelam GC, Hanhart CJ, Koornneef M** (1996) The *ELONGATED* gene of *Arabidopsis* acts independently of light and gibberellins in the control of elongation growth. *Plant J* **9:** 305-312

**Hansen D, Rick CM, Boyton JE** (1962) Linkage tests with mutants of Stubbe group III. *Tomato Genet Coop Rep* **12:** 28-29

**Hauge BM, Hanley SM, Cartinhour S, Cherry JM, Goodman HM, Koornneef M, Stam P, Chang C, Kempin S, Medrano L, Meyerowitz EM** (1993) An integrated genetic/RFLP map of the *Arabidopsis thaliana* genome. *Plant J* **3:** 745-754

**Hauser B, Cordonnier-Pratt M-M, Pratt LH** (1994) Differential expression of five phytochrome genes in tomato (*Lycopersicon esculentum* Mill.). *Plant Physiol* **105:** supp p 72

**Hauser BA, Cordonnier-Pratt M-M, Daniel-Vedele F, Pratt LH** (1995) The phytochrome gene family in tomato includes a novel subfamily. *Plant Mol Biol* **29:** 1143-1155

**Hiscox JD, Israelstam GF** (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. *Can J Bot* **57:** 1332-1334

**Hou Y, Von Arnim AG, Deng X-W** (1993) A new class of *Arabidopsis* constitutive photomorphogenic genes involved in regulating cotyledon development. *Plant Cell* **5:** 329-339

**Ichikawa H, Tanaka A, Tano S, Nagatani A** (1996) Isolation and characterization of photomorphogenic mutants of *Arabidopsis* which promote transduction of light-signal. In *Book of abstracts - 7th International Conference on* Arabidopsis *Research*, Norwich

**Inskeep WP, Bloom PR** (1985) Extinction coefficients of chlorophyll *a* and *b* in *N,N*-Dimethylformamide and 80% acetone. *Plant Physiol* **77:** 483-485

**Jacobsen SE, Olszewski NE** (1993) Mutations at the *SPINDLY* locus of *Arabidopsis* alter gibberellin signal transduction. *Plant Cell* **5:** 887-896

## *References*


**Jenkins GI, Christie JM, Fuglevand G, Long JC, Jackson JA** (1995) Plant responses to UV and blue light: biochemical and genetic approaches. *Plant Science* **112:** 117-138

**Johnson E, Bradley M, Harberd N, Whitelam GC** (1994) Photoresponses of light-grown *phyA* mutants of *Arabidopsis*. Phytochrome is required for the perception of daylength extentions. *Plant Physiol* **105:** 141-149

**Jones MG** Gibberellins and the *procera* mutant of tomato. *Planta* **172:** 280-284

**Joustra MK** (1970) Flower initiation in *Hyoscyamus niger* L. as influenced by widely divergent daylengths in different light qualities. *Meded Landbouwhogeschool Wageningen* **70-19:** 1-78

**Kendrick RE, Kronenberg GHM (eds)** (1994) *Photomorphogenesis in Plants*. Kluwer Academic Publishers, Dordrecht, The Netherlands

**Kerckhoffs LHJ, Kendrick RE, Whitelam GC, Smith H** (1992) Extension growth and anthocyanin responses of photomorphogenic tomato mutants to changes in the phytochrome photoequilibrium during the daily photoperiod. *Photochem Photobiol* **56:** 611-615

**Kerckhoffs LHJ, Van Tuinen A, Hauser BA, Cordonnier-Pratt M-M, Nagatani A, Koornneef M, Pratt LH, Kendrick RE** (1996) Molecular analysis of *tri*-mutant alleles in tomato indicates the *Tri* locus is the gene encoding the apoprotein of phytochrome B1. *Planta* **199:** 152-157

**Kerckhoffs LHJ, De Groot NAMA, Van Tuinen A, Schreuder MEL, Koornneef M, Kendrick RE** Physiological characterization of exaggerated-photoresponse mutants of tomato. *J Plant Physiol* (in press)

**Kerckhoffs LHJ, Schreuder MEL, Van Tuinen A, Koornneef M, Kendrick RE** Phytochrome control of anthocyanin biosynthesis in tomato seedlings: analysis using photomorphogenic mutants. *Photochem Photobiol* (in press)

**Kerr EA** (1958) Linkage relations of *l-2*. *Tomato Genet Coop Rep* **8:** 21

**Kerr EA** (1960) Segregation data for genes on chromosome 10. *Tomato Genet Coop Rep* **10:** 20-21

**Kerr EA** (1965) Identification of *high pigment*, (*hp*), tomatoes in the seedling stage. *Can J Plant Sci* **45:** 104-105

**Kerr EA** (1966) Another *xanthophyllous* (*Xa-3*) for chromosome 10. *Tomato Genet Coop Rep* **16:** 13

**Kerr EA** (1967) Position of *oli* on chromosome 10. *Tomato Genet Coop Rep* **17:** 33

**Kerr EA** (1977) Summary of gene linkage on chromosome 10. *Tomato Genet Coop Rep* **27:** 16-17

**Kerr EA** (1979a) A mutation to *tangerine* fruit color. *Tomato Genet Coop Rep* **29:** 25

**Kerr EA** (1979b) *High pigment*, (*hp*), is on chromosome 12. *Tomato Genet Coop Rep* **29:** 26

**Kerr EA** (1979c) *Yellow-green-2* (*yg-2*) may be on chromosome 12. *Tomato Genet Coop Rep* **29:** 27

**Kerr EA** (1981) *Yellow-green-2* (*yg-2*) and *auroid* (*aud*) are alleles. *Tomato Genet Coop Rep* **31:** 8

**Kerr EA** (1982a) Two- and three-point tests with *hy* and *nor* on chromosome 10. *Tomato Genet Coop Rep* **32:** 19

**Kerr EA** (1982b) Two- and three-point tests with *auv* on chromosome 10. *Tomato Genet Coop Rep* **32:** 20

**Kerr EA** (1982c) Three-point test of *sherry - sh* on chromosome 10. *Tomato Genet Coop Rep* **32:** 21

**Khurana JP, Poff KL** (1989) Mutants of *Arabidopsis thaliana* with altered phototropism. *Planta* **178:** 400-406

**Khush GS, Rick CM** (1966) At long last, a marker for chromosome 12. *Tomato Genet Coop Rep* **16:** 15

**Khush GS, Rick CM** (1968) Cytogenetic analysis of the tomato genome by means of induced deficiencies. *Chromosoma* **23:** 452-484

**Klein-Lankhorst R, Rietveld P, Machiels B, Verkerk R, Weide R, Gebhardt C, Koornneef M, Zabel P** (1991) RFLP markers linked to the root knot nematode resistance gene *Mi* in tomato. *Theor Appl Genet* **81:** 661-667

**Koornneef M, Kendrick RE** (1994) Photomorphogenic mutants of higher plants. In *Photomorphogenesis in Plants*. Kendrick RE, Kronenberg GHM (eds), Kluwer Academic Publishers, Dordrecht, pp 601-628

**Koornneef M, Stam P** (1992) Genetic analysis. In *Methods in* Arabidopsis *research*. Koncz C, Chua NH, Schell J (eds), World Scientific, Singapore, pp 83-99

**Koornneef M, Rollf E, Spruit CJP** (1980) Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) HEYNH. *Z Pflanzenphysiol* **100:** 147-160

**Koornneef M, Van der Veen JH, Spruit CJP, Karssen CM** (1981) Isolation and use of mutants with an altered germination behaviour in *Arabidopsis thaliana* and tomato. In *Induced Mutations - A Tool in Plant Breeding*. Vienna: International Atomic Energy Agency, pp 227-232

**Koornneef M, Cone JW, Dekens RG, O'Herne-Robers EG, Spruit CJP, Kendrick RE** (1985) Photomorphogenic responses of long hypocotyl mutants of tomato. *J Plant Physiol* **120:** 153-165

**Koornneef M, Bosma TDG, De Jong JH, Ramanna MS, Van der Veen JH** (1986) *Yellow-green* (*yg-6*) is an allele of *aurea*. *Tomato Genet Coop Rep* **36:** 8

**Koornneef M, Bosma TDG, Hanhart CJ, Van der Veen JH, Zeevaart JAD** (1990) The isolation and characterization of gibberellin-deficient mutants in tomato. *Theor Appl Genet* **80:** 852-857

**Koornneef M, Bade J, Hanhart CJ, Horsman K, Schel J, Soppe W, Verkerk R, Zabel P** (1993) Characterization and mapping of a gene controlling shoot regeneration in tomato. *Plant J* **3:** 131-141

**Koornneef M, Hanhart CJ, Kendrick CI, Kerckhoffs LHJ, Schreuder MEL, Van Tuinen A, Kendrick RE** (1996) Photomorphogenic mutants of tomato. In *Book of Abstracts - International Conference on Molecular Biology of Tomato*, Berkeley, California

**Kraepiel Y, Julien M, Cordonnier-Pratt M-M, Pratt L** (1994) Identification of two loci involved in phytochrome expression on *Nicotiana plumbaginifolia* and lethality of the corresponding double mutant. *Mol Gen Genet* **242:** 559-565

**Laemmli UK** (1970) Cleavage of structural protiens during the assembly of the thead of bacteriophage T4. *Nature* **227:** 680-685

*References*

**Lanahan MB, Ho TD** (1988) Slender barley: A constitutive gibberellin-response mutant. *Planta* **175:** 107-114

**Lazarova GI, Brandstädter J, Matsui M, Cordonnier-Pratt M-M, Pratt LH, Kendrick RE** (1996) In *Book of abstracts - International Conference on Molecular Biology of Tomato*, Berkeley, California

**Lesley JW, Lesley MM** (1956) Effect of seed treatments with X-ray and phosphorus 32 on tomato plants of first, second, and third generations. *Genetics* **41:** 575-588

**Lesley JW, Lesley MM** (1971) Further study of the *sherry* mutation induced by ionizing radiation. *Tomato Genet Coop Rep* **21:** 22-23

**Lesley JW, Lesley MM** (1980) Linkage of *sh* (*sherry*). *Tomato Genet Coop Rep* **30:** 26

**Li J, Nagpal P, Vitart V, McMorris TC, Chory J** (1996) A role for brassinosteroids in light-dependent development of *Arabidopsis*. *Science* **272:** 398-401

**Lichtenthaler HK, Wellburn AR** (1983) Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochem Soc Trans* **11:** 591-592

**Liharska T, Van der Eijken W, Gheijsen G, Zabel P** (1994) Mapping of tomato genes induced in a compatible root-knot nematode interaction. *Tomato Genet Coop Rep* **44:** 23-25

**Lin C, Ahmad M, Gordon D, Cashmore AR** (1995a) Expression of an *Arabidopsis* cryptochrome gene in transgenic tobacco results in hypersensitivity to blue, UV-A and green light. *Proc Natl Acad Sci USA* **92:** 8423-8427

**Lin C, Robertson DE, Ahmad M, Raibekas AA, Shuman Jorns M, Dutton PL, Cashmore AR** (1995b) Association of flavin adenine dinucleotide with the *Arabidopsis* blue-light receptor CRY1. *Science* **269:** 968-970

**Lipucci Di Paola M, Collina Genci F, Caltavuturo L, Tognoni F, Lercari B** (1988) A phytochrome mutant from tissue culture of tomato. *Adv Hort Sci* **2:** 30-32

**Liscum E, Briggs WR** (1995) Mutations in the NPH1 locus of *Arabidopsis* disrupt the perception of phototropic stimuli. *Plant Cell* **7:** 473-485

**Liscum E, Hangarter RP** (1991) *Arabidopsis* mutants lacking blue light-dependent inhibition of hypocotyl elongation. *Plant Cell* **3:** 685-694

**Liscum E, Young JC, Poff KL, Hangarter RP** (1992) Genetic separation of phototropism and blue light inhibition of stem elongation. *Plant Physiol* **100:** 267-271

**López-Juez E, Nagatani A, Tomizawa K-I, Deak M, Kern R, Kendrick RE, Furuya M** (1992) The cucumber long hypocotyl mutant lacks a light-stable PHYB-like phytochrome. *Plant Cell* **4:** 241-251

**Malhotra K, Kim S-T, Batschauer A, Dawut L, Sancar A** (1995) Putative blue-light photoreceptors from *Arabidopsis thaliana* and *Sinapis alba* with a high degree of sequence homology to DNA photolyase contain the two photolyase cofactors but lack DNA repair activity. *Biochemistry* **34:** 6892-6899

**Mancinelli AL** (1994) The physiology of phytochrome action. In *Photomorphogenesis in Plants*. Kendrick RE, Kronenberg GHM (eds), Kluwer Academic Publishers, Dordrecht, pp 211-269

**McArthur JW** (1934) Linkage groups in the tomato. *J Genetics* **29:** 123-133

**McCormac AC** (1993) *Photoregulation by the phytochrome family: A physiological study of transgenic plants*. PhD thesis, University of Leicester, UK

**McCormac A, Whitelam G, Smith H** (1992) Light-grown plants of transgenic tobacco expressing an introduced oat phytochrome A gene under the control of a constitutive viral promotor exhibit persistent growth inhibition by far-red light. *Planta* **188:** 173-181.

**McNellis TW, Deng X-W** (1995) Light control of seedling morphogenic pattern. *Plant Cell* **7:** 1749-1761

**Miller AJ, McGrath RB, Chua N-H** (1994) Phytochrome phototransduction pathways. *Annu Rev Genet* **28:** 325-349

**Misèra S, Müller AJ, Weiland-Heidecker U, Jürgens G** (1994) The *FUSCA* genes of *Arabidopsis*: Negative regulators of light responses. *Mol Gen Genet* **244:** 242-252

**Mochizuki T, Kamimura S** (1986) Allelism test of *high-pigment* phenotypes by the 'Yellow film method'. *Tomato Genet Coop Rep* **36:** 15-16

**Mohr H** (1994) Coaction between pigment systems. In *Photomorphogenesis in Plants*. Kendrick RE, Kronenberg GHM (eds), Kluwer Academic Publishers, Dordrecht, pp 353-373

**Moran R** (1982) Formulae for determination of chlorophyllous pigments extracted with *N,N*-Dimethylformamide. *Plant Physiol* **69:** 1376-1381

**Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15:** 473-497

**Mutschler MA** (1984) Inheritance and linkage of the '*Alcobaca*' ripening mutant in tomato. *J Amer Soc Hort Sci* **109:** 500-503

**Nagatani A, Yamamoto KT, Furuya M, Fukumoto T, Yamashita A** (1983) Production and characterization of monoclonal antibodies to rye phytochrome. *Plant Cell Physiol* **24:** 1143-1149

**Nagatani A, Yamamoto KT, Fukumoto T, Yamashita A** (1984) Production and characterization of monoclonal antibodies which distinguish different surface structures of pea (*Pisum sativum* cv. Alaska) phytochrome. *Plant Cell Physiol* **25:** 1059-1068

**Nagatani A, Chory J, Furuya M** (1991) Phytochrome B is not detectable in the *hy3* mutant of *Arabidopsis*, which is deficient in responding to end-of-day far-red light treatments. *Plant Cell Physiol* **32:** 1119-1122

**Nagatani A, Reed JW, Chory J** (1993) Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. *Plant Physiol* **102:** 269-277

**Neuhaus G, Bowler C, Kern R, Chua N-H** (1993) Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. *Cell* **73:** 937-952

**Odinot P, Pet G, Lindhout P** (1992) An $F_2$ between *Lycopersicon esculentum* cv. Allround and *L. pennellii* for mapping studies. *Tomato Genet Coop Rep* **42:** 27-29

**Oelmüller R, Kendrick RE** (1991) Blue light is required for survival of the tomato phytochrome-deficient *aurea* and the expression of four nuclear genes coding for plastidic proteins. *Plant Mol Biol* **16:** 293-299

**Oelmüller R, Kendrick RE, Briggs WR** (1989) Blue-light mediated accumulation of nuclear-encoded transcripts coding for proteins of the thylakoid membrane is absent in the phytochrome-deficient *aurea* mutant of tomato. *Plant Mol Biol* **13:** 223-232

**Ogden RC, Adams DA** (1987) Electrophoresis in agarose and acrylamide gels. In *Methods in enzymology*. Volume 152. Guide to molecular cloning techniques. Berger SL, Kimmel AR (eds), Academic Press Inc., San Diego, pp 61-88

**Parks BM, Quail PH** (1991) Phytochrome-deficient *hy1* and *hy2* long hypocotyl mutants of *Arabidopsis* are defective in phytochrome chromophore biosynthesis. *Plant Cell* **3:** 1177-1186

**Parks BM, Quail PH** (1993) *hy8*, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. *Plant Cell* **5:** 39-48

**Parks BM, Jones AM, Adamse P, Koornneef M, Kendrick RE, Quail PH** (1987) The *aurea* mutant of tomato is deficient in spectrophotometrically and immunochemically detectable phytochrome. *Plant Mol Biol* **9:** 97-107

**Paterson AH, Lin Y-R, Li Z, Schertz KF, Doebley JF, Pinson SRM, Liu S-C, Stansel JW, Irvine JE** (1995) Convergent domestication of cereal crops by independent mutations at corresponding genetic loci. *Science* **269:** 1714-1718

**Persson AR** (1960) Physiological and genetical investigations on chlorophyll mutants. *Tomato Genet Coop Rep* **10:** 27-28

**Peters JL, Van Tuinen A, Adamse P, Kendrick RE, Koornneef M** (1989) High pigment mutants of tomato exhibit high sensitivity for phytochrome action. *J Plant Physiol* **134:** 661-666

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

**Peters JL, Schreuder MEL, Verduin SJW, Kendrick RE** (1992) Physiological characterization of a high-pigment mutant of tomato. *Photochem Photobiol* **56:** 75-82

**Pratt LH** (1995) Phytochromes: differential properties, expression patterns and molecular evolution. *Photochem Photobiol* **61:** 10-21

**Pratt LH, Cordonnier-Pratt M-M, Hauser B, Caboche M** (1995) Tomato contains two differentially expressed genes encoding B-type phytochromes, neither of which can be considered an ortholog of *Arabidopsis* phytochrome B. *Planta* **197:** 203-206

**Quail PH** (1994) Phytochrome genes and their expression. In *Photomorphogenesis in Plants*. Kendrick RE, Kronenberg GHM (eds), Kluwer Academic Publishers, Dordrecht, pp 71-103

**Quail PH, Boylan MT, Parks BM, Short TW, Xu Y, Wagner D** (1995) Phytochromes: photosensory perception and signal transduction. *Science* **268:** 675-680

**Reed JW, Nagpal P, Poole DS, Furuya M, Chory J** (1993) Mutations in the gene for red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. *Plant Cell* **5:** 147-157

**Reeves AF** (1972) Additional linkage tests with 1-134. *Tomato Genet Coop Rep* **22:** 21

**Reeves AF, Boyton JT, Hernandez BG, Rick CM** (1966) Additional linkage tests with mutants of Stubbe's groups I, II, III and IV. *Tomato Genet Coop Rep* **16:** 23-27

**Reeves AF, Hernández-Bravo G, Zobel RW, Rick CM** (1967) Additional linkage tests with mutants of Stubbe's series. *Tomato Genet Coop Rep* **17:** 42-44

**Reid JB, Ross JJ** (1993) A mutant-based approach, using *Pisum sativum*, to understanding plant growth. *Int J Plant Sci* **154:** 22-34

**Reymond P, Short TW, Briggs WR, Poff KL** (1992) Light-induced phosphorylation of membrane protein plays an early role in signal transduction for phototropism in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **89:** 4718-4721

**Reynard GB** (1956) Origin of the Webb Special (Black Queen) tomato. *Tomato Genet Coop Rep* **6:** 22

**Rick CM** (1955) Linkage between *ag* and *h*. *Tomato Genet Coop Rep* **5:** 23

**Rick CM** (1956) The linkage relations of *ag*. *Tomato Genet Coop Rep* **6:** 25

**Rick CM** (1959) Allele test between *au* and *brac*. *Tomato Genet Coop Rep* **9:** 42

**Rick CM** (1974) High soluble-solids content in large-fruited tomato lines derived from a wild green-fruited species. *Hilgardia* **42:** 493-510

**Rick CM, Fobes JF** (1977) Linkage relations of some isozymic loci. *Tomato Genet Coop Rep* **27:** 22-24

**Rick CM, Zobel RW** (1970) Results of recent allele tests. *Tomato Genet Coop Rep* **20:** 50-52

**Rick CM, Khush GS, Andrásfalvy A** (1967) *Flecked dwarf*, a marker for 12L. *Tomato Genet Coop Rep* **17:** 45-46

**Rick CM, Reeves AF, Zobel RW** (1968) Inheritance and linkage relations of four new mutants. *Tomato Genet Coop Rep* **18:** 34-35

**Rick CM, Borgnino F, Quiros C, Meredith CP** (1974) Segregation and linkage relations of new EMS mutants. *Tomato Genet Coop Rep* **24:** 22-24

**Rick CM, Cisneros P, Chetelat RT, DeVerna JW** (1994) *Abg* a gene on chromosome 10 for purple fruit derived from *S. lycopersicoides*. *Tomato Gen Coop Rep* **44:** 29-30

**Robson PRH, Whitelam GC, Smith H** (1993) Selected components of the shade-avoidance syndrome are displayed in a normal manner in mutants of *Arabidopsis thaliana* and *Brassica rapa* deficient in phytochrome B. *Plant Physiol* **102:** 1179-1184

**Rüdiger W, Thümmler F** (1994) The phytochrome chromophore. In *Photomorphogenesis in Plants*. Kendrick RE, Kronenberg GHM (eds), Kluwer Academic Publishers, Dordrecht, pp 51-70

**Senger H, Schmidt W** (1994) Diversity of photoreceptors. In *Photomorphogenesis in Plants*. Kendrick RE, Kronenberg GHM (eds), Kluwer Academic Publishers, Dordrecht, pp 301-325

**Sharma R, López-Juez E, Nagatani A, Furuya M** (1993) Identification of photo-inactive phytochrome A in etiolated seedlings and photo-active phytochrome B in green leaves of the *aurea* mutant of tomato. *Plant J* **4:** 1035-1042

**Sharrock RA** (1996) Structure and function of the *Arabidopsis* phytochrome photo-receptor family. In *Book of Abstracts - 24rd Annual Meeting American Society for Photobiology*, Atlanta, Georgia

**Sharrock RA, Parks BM, Koornneef M, Quail PH** (1988) Molecular analysis of the phytochrome deficiency in an *aurea* mutant of tomato. *Mol Gen Genet* **213:** 9-14

**Smith H** (1995) Physiological and ecological function within the phytochrome family. *Annu Rev Plant Physiol Plant Mol Biol* **46:** 289-315

**Somers DE, Sharrock RA, Tepperman JM, Quail PH** (1991) The *hy3* long hypocotyl mutant of *Arabidopsis* is deficient in phytochrome B. *Plant Cell* **3:** 1263-1274

**Soost RK, Lesley JW** (1959) A further linkage test with the *aurea* (*au*) mutant. *Tomato Genet Coop Rep* **9:** 46-47

**Soressi GP** (1975) New sponteneous or chemically-induced fruit-ripening tomato mutants. *Tomato Genet Coop Rep* **25:** 21-22

**Stam P** (1993) Construction of integrated genetic linkage maps by means of a new computer package: JOINMAP. *Plant J* **3:** 739-744

**Stubbe H** (1957) Mutanten der Kulturtomate *Lycopersicon esculentum* Miller I. *Die Kulturpflanze* **V:** 190-220

**Tanksley SD** (1993) Linkage map of tomato (*Lycopersicon esculentum*). In *Genetic maps*. O'Brien SJ (ed), Cold Spring Harbor Laboratory Press, New York, pp 6.39

**Tanksley SD, Ganal MW, Prince JP, DeVincente MC, Bonier MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Roder MS, Wing RA, Wu W, Young ND** (1992) High density molecular linkage maps of the tomato and potato genomes. *Genetics* **132:** 1141-1160

**Tanksley SD, Ganal MW, Martin GB** (1995) Chromosome landing: a paradigm for map-based cloning in plants with large genomes. *Trends Genet* **11:** 63-68

**Terry MJ, Kendrick RE** The *au* and *yg-2* mutants of tomato are deficient in phytochrome chromophore synthesis. *J Biol Chem* **271:** 21681-21686

**Terry MJ, Wahleithner JA, Lagarias JC** (1993) Biosynthesis of the plant photoreceptor phytochrome. *Arch Biochem Biophys* **306:** 1-15

**Tigchelaar EC, Barman RJ** (1978) Linkage of the *non-ripening* (*nor*) and *uniform ripening* (*u*) genes. *Tomato Genet Coop Rep* **28:** 20

**Tigchelaar EC, Tomes ML, Kerr EA, Barman RJ** (1973) A new fruit-ripening mutant, *non-ripening* (*nor*). *Tomato Genet Coop Rep* **23:** 33-34

**Thompson AE** (1961) A comparison of fruit constituents of normal and high pigment tomatoes. *Proc Amer Soc Hort Sci* **78:** 464-473

**Tsuge T, Frances S, Chamovitz D, Matsui M, Deng X-W** (1996) Isolation and characterization of *Arabidopsis COP1* homologous genes in both mono- and dicotyledonous plants. In *Book of Abstracts - 7th International Conference on* Arabidopsis *Research*, Norwich

**Van den Bulk R, Löffler HJM, Lindhout WH, Koornneef M** (1990) Somaclonal variation in tomato: effect of explant source and a comparison with chemical mutagenesis. *Theor Appl Genet* **80:** 817-825

**Van der Beek JG, Verkerk R, Zabel P, Lindhout P** (1992) Mapping strategy for resistance genes in tomato based on RFLPs between cultivars: *Cf9* (resistance to *Cladosporium fulvum*) on chromosome 1. *Theor Appl Genet* **84:** 106-112

**Van der Biezen EA, Overduin B, Nijkamp HJJ, Hille J** (1994) Integrated genetic map of tomato chromosome 3. *Tomato Genet Coop Rep* **44:** 8-10

**Van Ooijen JW** (1994) Drawmap: a computer program for drawing genetic linkage maps. *J Hered* **85:** 66

**Van Tuinen A, Kerckhoffs LHJ, Nagatani A, Kendrick RE, Koornneef M** (1995a) Far-red light-insensitive, phytochrome A-deficient mutants of tomato. *Mol Gen Genet* **246:** 133-141

**Van Tuinen A, Kerckhoffs LHJ, Nagatani A, Kendrick RE, Koornneef M** (1995b) A temporarily red light-insensitive mutant of tomato lacks a light-stable, B-like phytochrome. *Plant Physiol* **108:** 939-947

**Van Tuinen A, Hanhart CJ, Kerckhoffs LHJ, Nagatani A, Boylan MT, Quail PH, Kendrick RE, Koornneef M** (1996) Analysis of phytochrome-deficient *yellow-green-2* and *aurea* mutants of tomato. *Plant J* **9:** 173-182

**Van Wordragen MF, Weide RL, Coppoolse E, Koornneef M, Zabel P** (1996) Tomato chromosome 6: A high resolution map of the long arm and construction of a composite integrated marker-order map. *Theor Appl Genet* **92:** 1065-1072

**Vince-Prue D** (1994) The duration of light and photoperiodic responses. In *Photomorphogenesis in Plants.* Kendrick RE, Kronenberg GHM (eds), Kluwer Academic Publishers, Dordrecht, pp 447-490

**Von Wettstein-Knowles P** (1968) Mutations affecting anthocyanin synthesis in the tomato. *Hereditas* **60:** 317-346

**Wagner D, Fairchild CD, Kuhn RM, Quail PH** Chromophore-bearing $NH_2$-terminal domains of phytochromes A and B determine their photosensory specificity and differential light lability. *Proc Natl Acad Science USA* **93:** 4011-4015

**Wei N, Deng X-W** (1992) *COP9*: A new genetic locus involved in light-regulated development and gene expression in *Arabidopsis*. *Plant Cell* **4:** 1507-1518

**Wei N, Chamovitz DA, Deng X-W** (1994a) *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. *Cell* **78:** 117-124

**Wei N, Kwok SF, Von Arnim AG, Lee A, McNellis TW, Piekos B, Deng X-W** (1994b) *Arabidopsis COP8, COP10* and *COP11* genes are involved in repression of photo- morphogenic development in darkness. *Plant Cell* **6:** 629-643

**Weide R, Van Wordragen MF, Klein-Lankhorst R, Verkerk R, Hanhart C, Liharska T, Pap E, Stam P, Zabel P, Koornneef M** (1993) Integration of the classical and molecular linkage maps of tomato chromosome 6. *Genetics* **135:** 1175-1186

**Weller JL, Nagatani A, Kendrick RE, Murfet IC, Reid JB** (1995) New *lv* mutants of pea are deficient in phytochrome B. *Plant Physiol* **108:** 525-532

**Weller JL, Terry MJ, Rameau C, Reid JB, Kendrick RE** (1996) The phytochrome deficient *pcd1* mutant of pea is unable to convert heme to biliverdin IXα. *Plant Cell* **8:** 55-67

**Whitelam GC, Harberd NP** (1994) Action and function of phytochrome family members revealed through the study of mutant and transgenic plants. *Plant Cell Environ* **17:** 615-625

**Whitelam GC, Smith H** (1991) Retention of phytochrome-mediated shade avoidance responses in phytochrome deficient mutants of *Arabidopsis*, cucumber and tomato. *J Plant Physiol* **139:** 119-125

**Whitelam GC, Johnson E, Peng J, Carol P, Anderson ML, Cowl JS, Harberd NP** (1993) Phytochrome a null mutants of Arabidopsis display a wild-type phenotype in white light. *Plant Cell* **5:** 757-768

**Young PA, McArthur JW** (1947) Horticultural characters of tomatoes. *Texas Agricultural Experiment Station Bulletin* No. **698**

*References*

**Young JC, Liscum E, Hangarter RP** (1992) Spectral-dependence of light-inhibited hypocotyl elongation in photomorphogenic mutants of *Arabidopsis*: evidence for a UV-A photosensor. *Planta* **188:** 106-114

**Zobel RW, Alán JJ, Opeña, Rick CM** (1969) Further tests with Dr. Stubbe's mutants. *Tomato Genet Coop Rep* **19:** 30-31

# Samenvatting

Licht is voor planten de belangrijkste bron van energie en informatie. Een kiemend zaad verandert zijn groeipatroon op het moment dat het door het grondoppervlak heen breekt van een aan het donker aangepaste (geëtioleerde) zaailing in een aan het licht aangepaste groene fotosynthetisch actieve plant. Dit proces heet deëtiolering en maakt deel uit van de fotomorfogenese, waartoe alle morfologische invloeden door licht gerekend worden. Deëtiolering behelst de remming van hypocotylstrekking, het openen van de apicale haak, het uitvouwen van de cotylen, de ontwikkeling van chloroplasten en de aanmaak van anthocyaan. Om de hoeveelheid, duur, kwaliteit en richting van licht te bepalen, bezitten planten verschillende groepen pigmenten (fotoreceptoren genoemd). De nu bekende fotoreceptoren zijn de blauw licht (B) absorberende cryptochromen en de rood (R)/verrood (FR) licht absorberende fytochromen. Daarnaast bestaat waarschijnlijk nog een UV-B/UV-A specifieke fotoreceptor. Mutanten waarin één van deze fotoreceptoren niet meer aanwezig is of niet meer functioneert, kunnen worden gebruikt om het aandeel van de verschillende fotoreceptoren en hun interactiepatroon in de regulatie van de fotomorfogenese te bepalen.

Van de verschillende fotoreceptoren is fytochroom het best gekarakteriseerd. Fytochroom komt voor in twee verschillende vormen: de R absorberende vorm (Pr) en de FR absorberende vorm (Pfr). Pr wordt aangemaakt in het donker en na belichting met R omgezet in Pfr. Pfr wordt op zijn beurt weer omgezet in Pr na belichting met FR. De vorming van Pfr leidt, via een keten van reacties tot een groot aantal fysiologische responsen, zoals zaadkieming, deëtiolering, schaduwmijding en de aanzet tot bloei.

Fytochroom bestaat uit een eiwit, waaraan een chromofoorgroep, bestaand uit vier pyrrolkernen, gekoppeld is. Deze laatste groep is verantwoordelijk voor het licht absorberend vermogen van fytochroom. In hogere planten worden de fytochroomeiwitten gecodeerd door een kleine gen familie, terwijl wordt aangenomen dat alle eiwitten dezelfde chromofoor dragen. Fytochromen kunnen worden geclassificeerd in twee typen: een licht

labiel type, waarin het Pfr na vorming door belichting snel wordt afgebroken en een relatief licht stabiel type. Tot het eerste type behoort fytochroom A (phyA) terwijl alle andere tot dusver gekarakteriseerde fytochromen tot het tweede type lijken te behoren. Het expressie niveau van de verschillende fytochroom genen, althans in tomaat, verschilt in tussen genen, per gen gedurende de levenscyclus van de plant en het weefsel/orgaan waar het gen tot expressie komt.
In de tomaat, die voor dit onderzoek als model plant gebruikt is, bestaat de fytochroom gen familie uit tenminste 9 en mogelijk zelfs 13 genen. Vijf van deze genen zijn gekarakteriseerd en hun expressiepatroon is vastgesteld. Het is duidelijk dat fotomorfogenese een ingewikkeld proces is, waarbij de opheldering van de wegen die leiden tot de fotomorfogenetische responsen om specifieke fotomorfogenetische mutanten vraagt. Deze zogenoemde genetische aanpak wordt gezien als een van de meest veelbelovende benaderingen in het onderzoek naar de fotomorfogenese in planten.

Zaden, afkomstig van uit chemisch behandeld zaad opgekweekte planten, zijn onder continu R en B getest op het voorkomen van zaailingen met een afwijkend uiterlijk (mutant) ten opzichte van een normale zaailing (wildtype). Hoofdstuk 2 beschrijft de isolatie en karakterisering van twee mutanten, die respectievelijk in R en B een iets langer hypocotyl hebben dan het wildtype (WT). De twee mutanten bleken allelen van dezelfde locus op chromosoom 10 te zijn. Verdere analyse toonde aan dat de mutanten totaal ongevoelig zijn voor FR en zijn daarom *fri*$^{1}$ en *fri*$^{2}$ (far-red light insensitive) genoemd. Met behulp van antilichamen is aangetoond dat in geëtioleerde *fri* zaailingen het fytochrooom A eiwit (PHYA) afwezig is, net als het grootste deel van het spectrofotometrisch aantoonbare fytochroom. Fytochroom B eiwit (PHYB) is aanwezig in normale hoeveelheden en spectrofotometrisch is een kleine hoeveelheid licht stabiel fytochroom aantoonbaar. Volwassen *fri* planten geven een normale strekkingsresponse na een eind van de dag FR (EODFR) behandeling, wat wijst op het normaal functioneren van stabiele fytochromen.

In hoofdstuk 3 (en deels in hoofdstuk 6) worden vier mutanten beschreven, die onder continu R een lang hypocotyl hebben en hierin lijken op de phyB deficiënte mutanten, die al eerder in andere plantensoorten gevonden waren. De vier mutanten, die allel bleken te zijn, verschillen kwantitatief of kwalitatief van het WT in PHYB1 eiwit of mRNA. De lokalisatie van het phyB1 gen en het *tri* mutant gen op dezelfde plaats op chromosoom 1 (Hoofdstuk 5) bevestigde dat de twee loci identiek zijn. In tegenstelling tot de phyB mutanten van andere plantensoorten, zijn de vier *tri* mutanten alleen ongevoelig voor R gedurende de eerste twee dagen na overgang van het donker naar R en reageren ze op een EODFR en een met verrood verrijkte daglicht behandeling. Ook in wit licht zijn de *tri* mutanten, die ten opzichte van het WT iets langgerekter zijn, veel minder extreem dan de phyB deficiënte mutanten in andere plantensoorten. De fytochroom familie van tomaat bezit echter twee phyB genen. De *tri* mutanten laten zien dat schaduwmijding, waarvan gedacht werd dat het een exclusief door phyB gereguleerd proces is, in de tomaat door meer dan één licht stabiel fytochroom gecontroleerd wordt.

Hoewel de *aurea* (*au*) mutant al veelvuldig voor fotomorfogenetisch onderzoek was gebruikt, was de precieze aard van de mutatie niet bekend. Dit laatste gold ook voor de in fenotype met de *au* mutant vergelijkbare *yellow-green-2* (*yg-2*) mutant. Lang werd gedacht dat het phyA deficiënte mutanten betrof, maar dit rijmde niet met alle resultaten. De isolatie van de phyA deficiënte *fri* mutanten (Hoofdstuk 2), die bovendien een fenotype hebben dat afwijkt van dat van de *au* en *yg-2* mutanten, rechtvaardigde de twijfels over de aard van de mutaties. In hoofdstuk 4 wordt aangetoond dat geëtioleerde zaailingen van de *yg-2* mutant ongeveer 25% van het WT niveau aan PHYA hebben, terwijl het PHYB gehalte normaal is. De *yg-2* mutant lijkt ook hierin op de *au* mutant. Zowel de *au* als *yg-2* mutant werden gekruist met een tomatenlijn die, naast de eigen fytochroom genen, het gen voor haver fytochroom A3 (*PhyA-3*) tot expressie brengt. Dit leverde echter geen herstel van het WT fenotype op. Met antilichamen werd aangetoond dat in zowel de *au,PhyA-3* als *yg-2,PhyA-3* dubbelmutant het haver

fytochroom A3 eiwit aanwezig is. Spectrofotometrische analyse wees echter uit dat dit fytochroom, evenals het resterende tomaat fytochroom, niet actief is. Dit is in overeenstemming met het aanname dat beide mutanten verstoord zijn in de biosynthese van de chromofoor.

In hoofdstuk 5 zijn de vijf gekarakteriseerde fytochroom genen van tomaat in kaart gebracht op de RFLP kaart. De positie van de *yg-2* mutant op chromosoom 12 is bevestigd en de klassieke kaart van chromosoom 12 herzien. De positie van de phyA deficiënte *fri* mutanten op chromosoom 10 werd verfijnd en de klassieke kaart van dit chromosoom is eveneens herzien. De positie van het *PhyA* gen is niet te onderscheiden van die van de *fri* locus. De *tri* mutant is, zowel op de klassieke als RFLP kaart, in kaart gebracht. Zoals verwacht werd het *PhyB1* gen op dezelfde plaats gelokaliseerd. Van mutanten met een *high pigment* (*hp*) fenotype, dat fytochroom responsen versterkt, zijn er meerdere beschreven. Allelie toetsen hebben bevestigd dat de *hp-2* mutant niet allel is aan eerder beschreven *hp* mutanten. Bovendien werd een *hp-2* allel geïdentificeerd dat een sterker fenotype geeft. Het *hp-2* gen werd op chromosoom 1, op zowel de klassieke als RFLP kaart, gelokaliseerdn en bleek zeer nauw gekoppeld te zijn aan het *PhyB1=tri* locus.

Hoofdstuk 6 bevat een algemene discussie waarin de resultaten uit dit proefschrift worden samengevat en gerelateerd aan fotomorfogenetische mutanten in andere plantensoorten. Tevens worden enkele kandidaat mutanten voor andere fytochromen, waarvan de karakterisering o.a. door gebrek aan specifieke antilichamen niet voltooid is, besproken en de richting waarin het onderzoek wordt voortgezet aangegeven.

# Nawoord

Twee en een half jaar over tijd is het er dan toch nog van gekomen en zit ik het nawoord van mijn proefschrift te schrijven om al die mensen die erbij betrokken zijn geweest te bedanken. Omdat ik de afgelopen jaren op drie verschillende vakgroepen heb rondgelopen is het een hele waslijst geworden.

Te beginnen met de vakgroep Erfelijkheidsleer waar het allemaal begon onder de bezielende leiding van mijn promotor Maarten Koornneef. Maarten: ik geloof niet dat een OIO zich een betere promotor kan wensen dan jou. Je had altijd tijd om wat voor vragen dan ook te beantwoorden en literatuur belandde automatisch op mijn bureau. Het was wel eens moeilijk om je de resultaten van een proef of het concept van een artikel gisteren al te geven, maar het was dan ook altijd vandaag al nagekeken en van commentaar voorzien. Als trendsetter in het genetisch/fysiologisch fytochroom onderzoek ben je in andere zaken zeer behoudend. Ik weet dat ik je wel eens angstig gemaakt heb als wetenschapper niet serieus genomen te worden, omdat ik mijn seminars graag met wat minder serieuze dia's begin en ook mijn meer persoonlijk getinte alternatief voor het curriculum vitae voor dit proefschrift moest ik naar herschrijven, omdat dit nu eenmaal gortdroog hoort te zijn.

De tweede constante erfelijkheidsfactor is analiste Corrie Hanhart geweest. Corrie, mijn eigen Vakgroeps Story tijdens het tomatensnijden en mijn verblijf in Japan, die voor mij zelfs enkele malen haar grenzen verlegde en na 16.30 uur, zelfs tot 24.00 uur en op zaterdag meehielp mijn proeven tot een goed einde te brengen.

Verder waren er mijn enige student Adri Peters, die gegevens voor een artikel verzamelde dat wat minder goed binnen dit proefschrift paste en nog op de planken ligt en mijn enige stagiaire Sonia Benito, van wie ik leerde dat je als vrouw 's zomers onmogelijk met ongeschoren benen in korte broek of rok kunt rondlopen.

De sfeer in de Botgen groep heb ik altijd als zeer prettig ervaren, mede dankzij mijn collega's Anne-marie, Herman (het bier van de band smaakte heerlijk na een dagje zweten in de kas), Karen, Wim, Ton, Bertrand en Isabelle (I should have practised my French with the two of you), Carlos (I

loved to discuss the profit or non-profit of learning Dutch and your endless supply of tangerines) en Hetty.

Natuurlijk waren daar ook de mannen van de tuin: Henk Kuiper, Jan Laurens, Willem Blijderveen en Gerrit van IJmeren, die zorg droegen voor de groei en bloei van de eindeloze hoeveelheid tomaten die ik in de loop der jaren heb verbruikt; die de tuinslang voor mij uitrolden zodat ik wat neer aan acrobatiek kon doen en voorspelden dat ik die schattige babymuisjes, die in de kas rondliepen, zou laten vermoorden op het moment dat ze de topjes uit mijn zaailingen hadden gegeten.

Verder bedank ik de rest van de collegae van de vakgroep Erfelijkheidsleer voor de gezellige koffie- en theepauzes, die vanwege de heerlijke zitgelegenheid in de zomer wel eens wat langer duurden dan de bedoeling was.

De volgende vakgroep waar ik heel wat tijd heb doorgebracht is de vakgroep Plantenfysiologie, waar mijn co-promotor Dick Kendrick huist. Dick, datgene wat ik over Maarten schreef geldt ook voor jou, een betere co-promotor dan jou kan ik me niet voorstellen. Met eindeloos geduld wist je de meer complexe fytochroom zaken uit te leggen totdat wij simpele genetici het ook begrepen; je gaf me de gelegenheid een paar maanden te werken op je andere lab in Japan, een land waarvan de tegenstelling tussen eeuwenoude cultuur en moderne maatschappij een enorme indruk heeft achtergelaten en ik profiteerde mee van jouw inzet voor betere doka's en belichtingskasten bij de verhuizing van het "Schip van Blaauw" naar "De Banaan". Chris, jouw naam mag ook wel eens genoemd worden. Als stille vennoot van Dick lees je alle artikelen nog eens extra door op zinnen waarvan nog mooier Engels gemaakt kan worden. De deur van jullie huis stond altijd open voor spoedbestellingen van artikelen en aanverwante zaken en niet te vergeten de gezellige borrelavondjes.

Het extra paar handen van Mariëlle Schreuder was altijd aanwezig om te assisteren bij het inrichten van de belichtingskasten en de uitvoering van proeven.

Huub, net als ik heb ook jij geprofiteerd van de samenwerking die er al tussen Maarten en Dick bestond. Hoewel we na een gemeenschappelijke

vliegende start ieder onze eigen weg zijn gegaan, heeft het ons samen heel wat artikelen opgeleverd.

Natuurlijk waren ook hier mijn plantjes niet in leven gebleven zonder de goede zorgen van fytotron medewerkers Leen Peterse en Henk Melissen.

Ook op deze vakgroep waren de koffie- en theepauzes niet zo gezellig geweest zonder de mensen die ik hier niet bij name genoemd heb.

Als laatste is de vakgroep Moleculaire Biologie aan de beurt, waar ik, hoewel met tussenpozen, alles bij elkaar genomen toch gauw een jaartje heb doorgebracht. Ruud, zonder jou had ik de RFLP techniek nooit zo vlug onder de knie gekregen, maar ik beken bij deze dat ik, ondanks gedegen training, nooit verder ben gekomen dan 32 DNA isolaties per dag.

De andere (ex)leden van de tomatengroep Guusje, Tsveta, Rob, Monique, Jan en Pim bedankt voor jullie adviezen na het vertrek van Ruud en de prettige werksfeer op het lab.

Verder niet te vergeten dank aan al die mensen bij wie ik altijd phosphor imager schermen kwam bietsen voor mijn hybridisatiesessies.

And last but not least my American/French connection Lee and Marie-Michèle, without your probes and genuine interest in the progress of the mapping chapter 5 would not have existed.

Rest mij nog te zeggen dat ik met plezier terugkijk op de periode waarin ik aan dit proefschrift heb gewerkt.

Ageeth

## Curriculum vitae

Ageeth van Tuinen werd op 19 augustus 1964 geboren in Zutphen. Na de lagere school in Brummen doorlopen te hebben, werd haar opleiding in 1976 voortgezet aan het Stedelijk Lyceum en school voor HAVO te Zutphen, wat in 1982 resulteerde in het behalen van het VWO-ß diploma. Hierna toog ze naar de toenmalige Landbouwhogeschool in Wageningen, waar ze voor de studie plantenveredeling koos. Na afstudeervakken gelopen te hebben op de Vakgroep Tuinbouw (*In vitro* cultuur), het voormalig Instituut voor de Veredeling van Tuinbouwgewassen (nu onderdeel van CPRO-DLO; plantenveredeling) en de vakgroepen Erfelijkheidsleer/Plantenfysiologisch Onderzoek (PFO; Genetica) werd in september 1988 de bul aan haar uitgereikt. Om later in de gelegenheid te zijn eventueel het onderwijs in te gaan, besloot ze er nog een jaar studie aan het STOAS aan vast te knopen en behaalde in juni 1989 haar tweedegraads akte voor het middelbaar en hoger agrarisch onderwijs. Per 1 juli 1990 begon ze als OIO (gesubsidieerd door BION/NWO) op de vakgroep Erfelijkheidsleer onder leiding van Prof. dr. ir. Maarten Koornneef en dr. Dick Kendrick van de vakgroep PFO aan de speurtocht naar nieuwe fotomorfogenese mutanten bij de tomaat en de verdere karakterisering van oude "bekenden", wat uiteindelijk heeft geleid tot het schrijven van dit proefschrift. Per 1 januari 1997 gaat ze als post-doc meedraaien in de groep van Dr. Höfte aan het INRA te Versailles, die moleculair genetisch onderzoek doet naar het celstrekkings mechanisme in *Arabidopsis* en de controle hiervan door licht.