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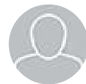

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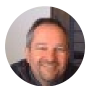

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Euphytica **79**: 227–??, 1994.
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Photomorphogenic mutants of tomato

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Key words: Lycopersicon esculentum, photomorphogenesis, phytochrome, signal transduction, chromophore, *aurea*

Abstract

Photomorphogenesis of tomato is being studied with the aid of mutants which are either modified in their photoreceptor composition or in their signal transduction chain(s). Several mutants affecting the phytochrome family of photoreceptors, some of which appear deficient for specific genes encoding phytochrome apoproteins have been isolated. In addition, other mutants, including transgenic lines overexpressing phytochrome A, exhibit exaggerated photomorphogenesis during de-etiolation. Anthocyanin biosynthesis and plastid development are being used as model systems for the dissection of the complex interactions among photomorphogenic photoreceptors and to elucidate the nature of their transduction chains.

Introduction

Photomorphogenesis is the process by which light regulates aspects of plant growth and development (Kendrick & Kronenberg, 1994). During the life cycle of a plant, light has been shown to play an important role in: (i) the photocontrol of germination; (ii) the process of de-etiolation, which results in the transition from the strategy of dark-adapted growth, while below the ground (typified by poorly developed leaves and rapid elongation growth while living heterotrophically on the seed food reserves), to the strategy of light-adapted growth, as a green photosynthetically self-sufficient seedling; (iii) shade avoidance (near-neighbour detection) due to the perception of change in spectral quality of the light environment as a consequence of transmission and reflectance from other plants. To achieve these and other light-regulated processes plants utilize at least three classes of photoreceptors: phytochromes which absorb predominantly in the red (R) and far-red (FR) region of the spectrum, blue light (B)/UV-A photoreceptor(s) and UV-B photoreceptor(s). Of these photoreceptors the phytochromes, which exist in two photo-interconvertible forms, one R-absorbing (Pr) and the other FR-absorbing (Pfr), are the most extensively studied. Phytochromes are encoded by a small gene family in all plant species so far studied (Quail, 1994). In *Arabidopsis* five genes have been described *PHYA*, *PHYB*, *PHYC*, *PHYD* and *PHYE*. These genes encode the apoproteins PHYA, PHYB, PHYC, PHYD and PHYE, which after assembly to the tetrapyrrole chromophore result in the photoreversible holophytochromes phyA, phyB, phyC, phyD and phyE, respectively (Sharrock & Quail, 1989; Clack et al., 1994; Quail, 1994; Quail et al., 1994). In contrast, tomato has recently been found to have more than the five genes described for *Arabidopsis*, at least one of which is quite distinct from any previously described (Cordonnier-Pratt et al., 1994). Phytochrome responses can be subdivided into response modes on the basis of the amount of light required: very-low fluence responses (VLFRs); low fluence responses (LFRs);

high irradiance responses (HIRs). There is also evidence of interactions among the different classes of photoreceptors. It is our aim to unravel this complexity by studying photomorphogenic mutants, in which elements of the system are modified (and hopefully have a more simplified photomorphogenesis), using tomato (*Lycopersicon esculentum* Mill.) as a model species.

Photomorphogenic mutants

Deficiency mutants

Recessive mutants at the *aurea* (*au*) locus (Koornneef et al., 1981, 1985) located on chromosome 1 (Khush & Rick, 1968) have long hypocotyls and a marked reduction in chlorophyll content when grown in white light (WL). A mutant at a second locus, but with a similar phenotype has been described. This is called yellow green-2 (*yg-2*) (allelic with *auroid*) and is located on chromosome 12 (Burdick, 1958; Kerr, 1979, 1981; Rick et al., 1968).

At the seedling stage, compared to wild type (WT), the *au* mutant is characterized by a reduction in: (i) hypocotyl growth inhibition in WL, FR, R, B and UV-A (Koornneef et al., 1985; Adamse et al., 1988); (ii) chlorophyll and chloroplast development (Koornneef et al., 1985; Ken-Dror & Horwitz, 1990; Neuhaus et al., 1993), appearing to lack the VLFR component in the fluence-response curve for greening; (iii) anthocyanin content (Adamse et al., 1989); (iv) the photoregulation of the transcript levels of chlorophyll *a/b*-binding proteins of photosystem I and II, plastocyanin and subunit II of photosystem I (Sharrock et al., 1988; Oelmüller & Kendrick, 1991); (v) the photo-induction of enzymes, e.g. phenylalanine ammonia lyase (PAL), nitrate reductase (NR), nitrite reductase (NiR) and amylase (Goud et al., 1991; Becker et al., 1992; Goud & Sharma, 1994). This pleiotropic phenotype, coupled with a lack of phytochrome in etiolated *au*-mutant tissues is precisely that predicted for a phytochrome-deficient mutant. Another aspect of the *au* phenotype is its reduced germination in darkness compared to WT (Koornneef et al., 1985). The freshly harvested seeds, which are dormant, can be induced to germinate after treatment with a combination of chilling and nitrate (Georghiou & Kendrick, 1991). Moreover, exposure to continuous R, an effect which could be replaced by R pulses, led to an increase in germination of *au*-mutant seed batches. Therefore, functional phytochrome must be present since the effect of R pulses was reversible by FR pulses. However, no inhibitory effect of continuous FR was observed in older seed batches with appreciable dark germination, in contrast to WT which exhibits a strong FR inhibition of germination (Koornneef et al., 1985). Lipucci di Paola et al. (1988) have found a promotion of seed germination by FR for *au* mutants and suggested that this is the consequence of the absence of an inhibitory FR-HIR.

Adult WL-grown plants of both WT and the *au* mutant exhibit a quantitatively similar elongation growth response to end-of-day FR (EODFR) treatment (Adamse et al., 1988; López-Juez et al., 1990; Peters et al., 1992a) and changes in the R:FR photon ratio during the daily photoperiod (Whitelam & Smith, 1991; Kerckhoffs et al., 1992), indicating the presence of functional phytochrome in WL-grown *au*-mutant plants. However, Casal & Kendrick (1993) have presented evidence which suggests that the *au* mutant is less capable of detecting small changes in R:FR indicating that de-etiolated plants have a partially aberrant shade-detection mechanism. The yellow colour of the leaves and fruits indicates that greening continues to be defective in mature *au* mutant plants. Surprisingly, the *au*-mutant leaves show net photosynthesis rates comparable to WT, despite their reduced chlorophyll content (López-Juez et al., 1990; Becker et al., 1992).

Etiolated seedlings of the *au* mutant contain less than 5% (detection limit) of the spectrophotometrically detectable phytochrome found in WT seedlings (Koornneef et al., 1985), whereas WL-grown tissues (flower petals and Norflorazon-bleached leaves) contain about 60% of that detectable in the WT (Adamse et al., 1988). Spectrophotometrically active phytochrome has been extracted from WL-grown plants (López-Juez et al., 1990) and has been shown to be recognized by an antibody raised against a fragment of tobacco PHYB (Sharma et al., 1993). Although initial studies using antibodies raised against phyA from a monocot (Parks et al., 1987; Oelmüller et al., 1989) indicated a lack of PHYA in etiolated seedlings, Sharma et al. (1993) using an antibody raised against dicot phyA showed that there was about 20% of the WT level of PHYA present which was stable in R, indicating that it might not be converted to Pfr and is not degraded by the destruction process. This result is consistent with this phytochrome pool lacking a chromophore. Since it is probable that all phytochromes have the same chromophore it is quite likely that etiolated *au* seedlings are deficient in *all* phytochromes.

McCormac (1993) was successful in transforming an *au* line with the oat *PHYA3* gene. Despite demonstrating the presence of *PHYA3* mRNA in two independent transformants, she was unable to detect any PHYA3 apoprotein by Western blotting. This led to the conclusion that the *au* mutation influenced some process affecting mRNA translation and not chromophore biosynthesis. We have taken a different approach and crossed an oat *PHYA3* overexpressing line (Boylan & Quail, 1989) with both the *au* mutant and the phenotypically similar *yg-2* mutant. From the F_2 progeny we have selected the *au,PHYA3* and *yg-2,PHYA3* double mutants. These were both shown to accumulate PHYA3 apoprotein, yet essentially no rescue of their mutant phenotype was observed. This outcome is entirely consistent with a deficiency in availability of the phytochrome chromophore. We are currently engaged in a more systematic approach to study the biochemical nature of the *au* mutation based on the hypothesis that the lesion results in a modification of a step in chromophore (phytochromobilin) biosynthesis. Since the *au* mutant is one of the best physiologically characterized photomorphogenic mutants it is of great interest to know the precise nature of the lesion. Several attempts to rescue the *au* mutant by feeding chromophore precursors such as biliverdin have so far failed to provide direct evidence for the mutation lying in the chromophore pathway, despite the fact that this approach has been successful with chromophore mutants of *Arabidopsis* (Parks & Quail, 1991). The lack of rescue may be because the mutation lies at a step after biliverdin. The lack of rescue of WT seedlings treated with gabaculine, an inhibitor of tetrapyrrole biosynthesis and thus of phytochromobilin, means that there is as yet no positive control for these biliverdin feeding experiments in tomato (P.H. Quail, personal communication; R. Sharma, personal communication). If *au* is a chromophore mutant then the efficiency with which it can photosynthesize suggests that heme is not limiting. In this case the mutation might be expected to lie between heme and phytochromobilin.

The lack of responsiveness of the *au* mutant to both R and FR is indicative of a chromophore mutant on the basis of our knowledge of phytochrome action in *Arabidopsis* (Quail, 1994). In addition the recent characterization of mutants that have specifically lost responsiveness to either R or FR in tomato (see below), again suggests that it is very unlikely that the *au* mutation is specific for one phytochrome type. Chromophore mutants enable the effects of severe phytochrome deficiency to be studied, even where there are overlapping functions of different phytochromes in such processes as the inhibition of hypocotyl growth. In addition, since such mutants are considered to be slightly leaky, the phenotypic severity of the mutation can be manipulated by the degree of co-action with other photoreceptors. In tomato, under R alone the *au* and *yg-2* mutations are lethal, but by introduction of different quantities of B the severity of the mutant phenotype can be manipulated. This fact enables the *au* mutant to survive under WL and produce seed (Oelmüller & Kendrick, 1991). We have constructed the double mutant *au,yg-2* and it has a strong additive phenotype, being almost albino under certain light conditions at the seedling stage. However, despite the extreme phenotype, older seedlings do flower and set seed, albeit very inefficiently.

Since the *au* mutant is likely to be deficient in all phytochromes it has been impossible to use this mutant to assign function to the individual members of the phytochrome gene family in tomato. At least five phytochrome genes have been shown to be expressed in tomato (Hauser et al., 1994): *PHYA*, *PHYB1*, *PHYB2*, *PHYX* (which might be equivalent to *Arabidopsis PHYE*), and *PHYZ* (which is distinct from any previously described phytochrome). These genes show their own discrete pattern of expression throughout the life cycle of the plant. Southern analysis indicates further phytochrome-like sequences in the genome and therefore there is a high probability that additional phytochrome genes are expressed.

In a search for type specific phytochrome mutants we have recently selected several new long-hypocotyl mutants under low fluence rate (3 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) screens of B and R. Two of these mutants which are allelic were subsequently shown to be more or less completely blind to FR. Consequently we have called the locus FR insensitive (*fri*). These recessive mutants have been shown to lack the bulk pool of phytochrome in etiolated seedlings (predominantly phyA) and immunologically detectable PHYA (Van Tuinen et al., 1994). In addition, Northern analysis shows the *PHYA* mRNA is modified in the *fri* mutants. The *fri* locus has been mapped to chromosome 10, as has the *PHYA* gene. Since the *au* and *yg-2* mutants map to chromosomes 1 and 12, respectively, they cannot be specific phyA deficient mutants. Young WL-grown *fri*-mutant plants are almost indistinguishable from the WT, but one interesting observation is that on sunny days in the greenhouse older *fri*-mutant plants are prone to wilting, which results in retardation of growth.

Table 1. Summary of photomorphogenic mutants of tomato.

Genotype	WT	*au*	*yg-2*	*fri*	*tri*	*PHYA3*$^+$	*hp-1*	*hp-2*	*Ip*	*atv*
Chromosome No.		1	12	10	1	?	?	?	?	?
Hypocotyl inhibition										
FR	+	–	–	–	+	++	++	?	?	?
R	+	–	–	+	–/+	++	++	++	++	++
Adult plants										
Chlorophyll	++	+	+	++	++	+++	+++	+++	++	++
EODFR	+	+	+	+	+	+	+	+	+	+
Mutant type (putative)		(Chr$^-$)	(Chr$^-$)	phyA$^-$	phyB1$^-$	phyA3$^+$	(Resp$^+$)	(Resp$^+$)	(Resp$^+$)	(Resp$^+$)

WT = wild type; EODFR = end-of-day FR response; FR = far-red light; R = red light; ? = not determined; Chr$^-$ = chromophore biosynthesis deficiency; Resp$^+$ = response amplification.

Provisional experiments suggest that this is not due to abnormal behaviour of stomata.

Another group of recessive long-hypocotyl mutants selected under WL or low-fluence rate R were shown to be temporarily R insensitive (*tri*), being essentially blind to R during the first two days after transfer from darkness irrespective of their physiological age. Four alleles have been isolated and when examined by Western analysis one of these had none (below detection limit) and one had a reduced amount of a PHYB-like protein as compared to the WT. The other two alleles had polypeptides recognized by the PHYB antibody, but were of lower molecular masses than the PHYB-like protein in the WT. A Northern analysis reveals that two of the alleles have a modified *PHYB1* mRNA. We have mapped the *tri* locus to chromosome 1. The WL-grown plants of this mutant are slightly taller than the WT, but otherwise very similar. Furthermore, we have constructed the *fri,tri* double mutant which looks essentially the same as the *tri* mutant in WL, demonstrating that residual phytochromes can sustain a relatively *normal* photomorphogenesis. One interesting observation is the fact that these phyB1-deficient *tri* mutants exhibit a normal EODFR response. This result is in contrast to phyB-deficient mutants described so far in other species (Koornneef & Kendrick, 1994). Since phyB1 and phyB2 are closely related we hypothesize that they might both be able to regulate the EODFR response in tomato. Such a redundancy in the phytochrome system would explain why no constitutively tall tomato mutants have so far been found. The *fri,tri* double mutant is an ideal launch point for further mutagenesis and the selection of tall mutants deficient in the residual phytochromes.

Some properties of these deficiency mutants are summarized in Table 1.

Exaggerated response mutants

A spontaneous mutant at a high pigment (*hp*) locus was found as early as 1917 (Raynard, 1956). The monogenic recessive *hp-1* mutants are characterized by features such as dark-green foliage and immature fruit colour due to high chlorophyll levels (Sanders et al., 1975), higher lycopene and carotene content resulting in deep-red fruits (Thompson et al., 1962) and high levels of anthocyanin (Kerr, 1965; Von Wettstein Knowles, 1968). Mochizuki & Kamimura (1985) observed that *hp-1*-mutant hypocotyls had more anthocyanin than WT when grown in yellow light and used this as a selection criterion. Using continuous R we have selected several new, some very extreme, *hp-1* mutants (Adamse et al., 1989). Plant height is somewhat reduced in *hp-1* mutants (Peters et al., 1989, 1992a). Hypocotyl growth is more inhibited than that of WT when the seedlings are grown in R or yellow light (Mochizuki & Kamimura, 1985) and hypocotyl dry weight is lower than in WT when the seedlings are grown in WL (Von Wettstein Knowles, 1968). Thompson et al. (1962) reported that the seed germination of *hp-1* mutants was lower than WT and that the stems of *hp-1* mutant plants were more brittle resulting in a higher mortality. The pleiotropic nature of *hp-1* mutants suggest that they have a modification of a basic process affecting plant morphogenesis rather than being specific response mutants affecting pigment synthesis only.

There are also mutants which are similar in some aspects to the *hp-1* mutant phenotype, but map to different loci, such as *hp-2* (Soressi & Salamini, 1975), *atroviolatia* (*atv*) (Rick et al., 1968) and intensive pigment (*Ip*) (Rick, 1974). Furthermore, plants with *hp-1*-like characteristics at their seedling stage were obtained when high levels of the oat PHYA3 were expressed in tomato (Boylan & Quail, 1989). These transgenics all had short hypocotyls and more anthocyanin than WT at the seedling stage. Detailed fluence rate-response curves for anthocyanin biosynthesis in *hp-1* suggest that both the LFR and HIR components observed under R in WT (see below) are amplified (Peters et al., 1992b).

The *hp-1* mutant of tomato exhibits exaggerated phytochrome responses, whereas the phytochrome content of etiolated seedlings (predominantly phyA) and the physiological characteristics of the phytochrome system are similar to that in WT (Adamse et al., 1989; Peters et al., 1989). Therefore, there is so far no evidence to suggest that the *hp-1* mutant is a photoreceptor mutant. In contrast to WT, the *hp-1* mutant does not require co-action of the B photoreceptor and phytochrome for normal development and exhibits maximum anthocyanin synthesis and hypocotyl growth inhibition in R alone i.e. it mimics the action of B. On the basis of its recessive (loss-of-function) nature it is proposed that the phytochrome action in etiolated seedlings is under the constraint of the *HP-1*-gene product (HP-1) (Peters et al., 1992b). Both exposure to B and the *hp-1* mutation appear to result in reduction of HP-1 or its effectiveness. The exaggerated response of the *hp-1* mutant compared to WT fits the definition of *responsiveness amplification* proposed by Mohr (1994) to describe the amplification of a phytochrome response as a result of pre-irradiation which excites either the B photoreceptor or phytochrome. We propose that the *hp-1* mutation is associated with this amplification step in the phytochrome transduction chain. The phytochrome(s) deficient in the *au,hp-1* double mutant result in no, or severely reduced, anthocyanin accumulation indicating that at this stage of seedling de-etiolation the *au* mutation is more or less epistatic to *hp-1*. One interesting aspect of the phenotype of the *hp-1* mutants and *PHYA3* overexpressors is that under continuous low fluence rate FR both genotypes seem to abort their developmental program as the hypocotyls below the hooks appear to collapse and the hypocotyl breaks just below the cotyledons.

A study of the photoregulation of PAL, a key enzyme in flavonoid biosynthesis, showed a higher level in the *hp-1* mutant when compared to the *au* mutant, *au,hp-1* double mutant and WT level (Goud et al., 1991). Interestingly a R/FR reversible effect on PAL activity was shown in all these genotypes, indicating that etiolated seedlings of the *au* mutant do indeed contain some functional phytochrome. Goud & Sharma (1994) demonstrated that pulses of R are effective in the induction of amylase and NR activity in the WT and that the *hp-1* mutant exhibits an amplified response.

Adult plants of the *hp-1* and the *au,hp-1* double mutant show a quantitatively similar elongation response to reduction in R:FR photon ratio during the daily photoperiod and EODFR treatments (Kerckhoffs et al., 1992; Peters et al., 1992a). However, in WL-grown plants, the *hp-1* mutation appears to have a dwarfing effect in the *au,hp-1* double mutant particularly when fluorescent lighting (high R:FR) is used. In addition we have noticed that the *hp-1* phenotype is expressed during fruit development (dark-green pigmentation due to chlorophyll accumulation) in the *au,hp-1* double mutant. These results with WL-grown plants suggest that the *au* mutation is not completely epistatic to *hp-1* suggesting that the mutation is no longer limiting in mature plants, presumably due to the gradual accumulation of functional phytochromes.

Some properties of these exaggerated response mutants are summarized in Table 1.

Anthocyanin biosynthesis and plastid development as model systems

The *au*-mutant seedling has been used as a highly phytochrome-deficient starting material for investigation of phytochrome signal transduction. Neuhaus et al. (1993) micro-injected phyA into hypocotyl cells and elicited anthocyanin biosynthesis as well as partial plastid development. The lack of phytochrome in the *au* mutant enables these manipulative experiments to be carried out in the light. Their studies revealed evidence for two parallel pathways which were both induced by activation of one or more trimeric G-proteins. The pathway leading to partial plastid development was also induced by injection of calcium and calmodulin, whereas the pathway to anthocyanin biosynthesis was independent of calcium. Bowler et al. (1994) extended this work and provided evidence for cyclic GMP (cGMP) being an important intermediate. The anthocyanin response could be induced by micro-injection of cGMP alone and in the presence of

calcium could lead to the development of fully functional plastids indicating some cross talk between the signal transduction pathways involved in the regulation of gene expression during de-etiolation.

The responses taking place during de-etiolation show a strong tissue specificity in the hypocotyl. The anthocyanin production is restricted to the single subepidermal layer of cells, whereas all cells throughout the cortex have the capacity for plastid development into chloroplasts. Apart from guard cells the epidermal cells show neither response. We are engaged in an extensive study of anthocyanin biosynthesis under a 24-h irradiation schedule with different fluence rates of R. The WT response shows two components: a low fluence rate response and a HIR response at higher fluence rates. We have investigated these responses in the *fri* and *tri* mutants and have shown they are deficient in the low fluence rate and the HIR response components, respectively. Since the low fluence rate response, regulated by phyA, is only revealed at the very low light levels, anthocyanin accumulation under high fluence rate R, appears to be solely regulated by the phyB1, which is deficient in the *tri* mutant. Both response modes result in accumulation of anthocyanin in the same sub-epidermal cells. Overexpression of the *PHYA3* gene results in an increase in anthocyanin which is predominantly located in the same tissue-specific manner. Therefore in this transgenic line over-expressed *PHYA3* presumably maintains a higher than normal phyA pool, enhanced by the slower degradation of phyA3 than the endogenous tomato phyA. However, at medium to high R fluence rates the endogenous phyA in the WT is degraded before it has time to act. We are currently investigating the importance of calcium during photomorphogenesis of the hypocotyl, as well as attempting to develop a protoplast system amenable for study of the phytochrome signal transduction chain(s) *in vitro*. Using such a system, coupled with the mutants we have available and those under characterization, it is hoped that the complexities of phytochrome signal transduction will be unravelled.

Concluding remarks

The process of de-etiolation is not the same in all seedlings. If we take anthocyanin biosynthesis as an example of this point, in some species there is a very strong FR-HIR, while in tomato this is not the case. Nonetheless, in the same hypocotyl there is a strong FR-HIR for inhibition of elongation growth. Thus, comparative studies with different plant species will be very important. The study of tomato photomorphogenesis, especially as assisted by photomorphogenic mutants will consequently make a valuable complement to parallel studies with *Arabidopsis*.

Not only is the process of de-etiolation not the same in all seedlings, but it is quite complex. Perhaps the complexity exists because the selection pressure for this critical process in the life of a plant was so strong that several different photoreceptors, functioning in concert, have evolved to control it. In the laboratory, we have the opportunity to reveal response characteristics for excitation of selected photoreceptors which in nature never occurs. For example, the inhibition of elongation growth of a hypocotyl can be achieved by different wavelengths of light, in a number of different ways. The application of FR functions *via* the FR-HIR mode of phyA, whereas R functions *via* a R-LFR and a R-HIR in which both phyA and other phytochromes play a role. We propose that in nature none of these processes are saturated by the low light levels below the soil surface, but collectively they enable the selective advantage of perception of the light environment (soil surface) to be anticipated.

Acknowledgements

Supported by the Foundation for Life Sciences (SLW), formerly the Foundation for Fundamental Biological Research (BION) which is subsidized by the Netherlands Organization for the Advancement of Research (NWO), and by USDA NRICGP grants 9303030 to M-MC-P and 9300939 to LHP. We are most grateful to M.T. Boylan and P.H. Quail for providing seeds of the oat *PHYA3* overexpressing line discussed in this review.

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