Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Phytochrome Signal-Transduction: Characterization of Pathways and Isolation of Mutants

Simon A. Barnes, Ronaldo B. Quaggio and Nam-Hai Chua

Phil. Trans. R. Soc. Lond. B 1995 **350**, 67-74
doi: 10.1098/rstb.1995.0139

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. B* go to: **http://rstb.royalsocietypublishing.org/subscriptions**

This journal is © 1995 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Phytochrome signal-transduction: characterization of pathways and isolation of mutants

SIMON A. BARNES, RONALDO B. QUAGGIO AND NAM-HAI CHUA*

Laboratory of Plant Molecular Biology, The Rockefeller University, New York, New York 10021–6399 U.S.A.

SUMMARY

The study of phytochrome signalling has yielded a wealth of data describing both the perception of light by the receptor, and the terminal steps in phytochrome-regulated gene expression by a number of transcription factors. We are now focusing on establishing the intervening steps linking phytochrome photoactivation to gene expression, and the regulation and interactions of these signalling pathways. Recent work has utilized both a pharmacological approach in phototrophic soybean suspension cultures and microinjection techniques in tomato to establish three distinct phytochrome signal-transduction pathways: (i) a calcium-dependent pathway that regulates the expression of genes encoding the chlorophyll a/b binding protein (*CAB*) and other components of photosystem II; (ii) a cGMP-dependent pathway that regulates the expression of the gene encoding chalcone synthase (*CHS*) and the production of anthocyanin pigments; and (iii) a pathway dependent upon both calcium and cGMP that regulates the expression of genes encoding components of photosystem I and is necessary for the production of mature chloroplasts. To study the components and the regulation of phytochrome signal-transduction pathways, mutants with altered photomorphogenic responses have been isolated by a number of laboratories. However, with several possible exceptions, little real progress has been made towards the isolation of mutants in positive regulatory elements of the phytochrome signal-transduction pathway. We have characterized a novel phytochrome A (phyA)-mediated far-red light (FR) response in *Arabidopsis* seedlings which we are currently using to screen for specific phyA signal-transduction mutants.

1. INTRODUCTION

In 1986, at a Royal Society discussion meeting, our lab described *cis*-acting elements involved in the regulation of the expression of two photosynthetic genes: *CAB* and the gene encoding the small subunit of Rubisco (*RBCS*) (Nagy *et al.* 1986). These are the terminal steps in a signal-transduction pathway from the perception of light by phytochrome. Others have described in detail the characterization of the phytochrome molecule itself (for comprehensive reviews see Kendrick & Kronenburg 1994). Our current aim is to consider the intermediary steps of signal-transduction which link phytochrome photoactivation and gene expression. Neuhaus *et al.* (1993) and Bowler *et al.* (1994*a*,*b*) have established biochemically a number of positive regulatory components in the phytochrome phototransduction pathway and they have investigated the regulation of such pathways. In this paper we will review the conclusions from this work and describe a new screening strategy for the isolation of mutants in components of the phyA signalling pathway.

2. PHYTOCHROME SIGNAL TRANSDUCTION PATHWAYS

Neuhaus *et al.* (1993) devised a system to examine phytochrome responses at the single-cell level by microinjecting putative signal-transduction molecules into tomato hypocotyl cells. To avoid interference from endogenous phytochrome responses, microinjections were carried out using cells from the phytochrome-deficient *aurea* mutant of tomato. Etiolated *aurea* seedlings contain less than 5% of type I phytochrome levels found in the wild type (Parks *et al.* 1987; Sharrock *et al.* 1988). More recent work by Sharma *et al.* (1993) showed that although the amount of immunochemically detectable phyA protein in etiolated *aurea* seedlings was as high as 20% of that found in the wild type, this phyA was photoinactive. By contrast, the amount of phyB in *aurea* seedlings was not significantly altered. van Tuinen *et al.* (1995) have described new phyA-deficient mutants of tomato, denoted *fri* for FR-insensitive. These mutants have long hypocotyls in FR, and have no detectable phyA protein, although phyB protein levels appear to be normal. Unlike *aurea*, which has elongated hypocotyls and a pale green phenotype, these mutants show normal green leaf colour and growth characteristics in white light (W). The authors conclude that the *aurea* phenotype must be due to a deficiency in other phytochrome species or tetrapyrrole biosynthesis. However, this uncertainty in the basis for the *aurea* phenotype does not negate its use in microinjection systems. Microinjection of phyA should rescue only phyA mediated processes, therefore allowing the selective analysis of the effects of a single phytochrome species at the single cell level. Molecular processes which are not regulated by phyA should be unaffected. These results do not bear any relation to the gross morphology of the *aurea* mutant in W at the whole-

* Author to whom correspondence should be addressed.

© 1995 The Royal Society

The Royal Society is collaborating with JSTOR to digitize, preserve, and extend access to *Philosophical Transactions: Biological Sciences.* www.jstor.org

Downloaded from rstb.royalsocietypublishing.org

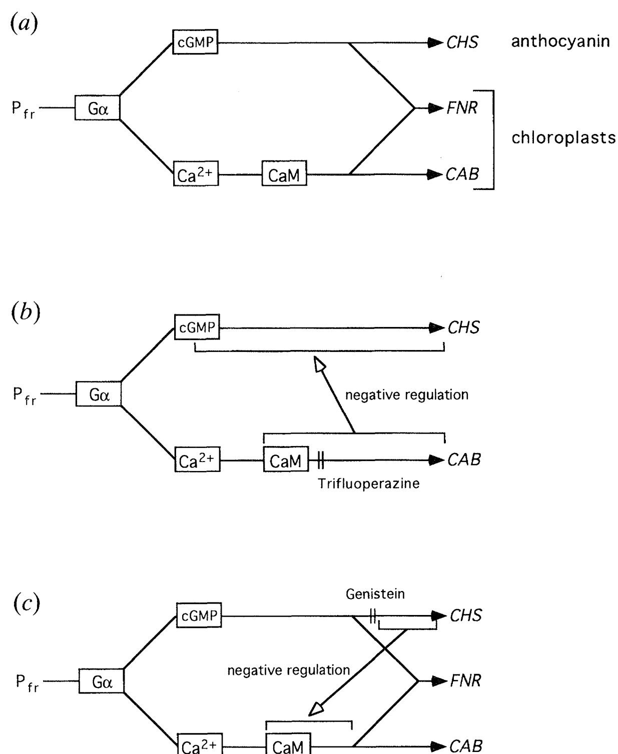


Figure 1. Phytochrome signal-transduction pathways and reciprocal control. (*a*) Phytochrome signal transduction is mediated by heterotrimeric G proteins, followed by calcium-, cGMP- and calcium/cGMP-dependent pathways. The cGMP-dependent pathway induces *CHS* expression and anthocyanin production. The calcium-dependent pathway induces the expression of *CAB* and genes encoding components of photosystem II. The calcium/cGMP-dependent pathway induces the expression of the gene encoding ferredoxin NADP+ oxidoreductase (*FNR*) and genes encoding components of photosystem I. Both the calcium- and calcium/cGMP-dependent pathways are necessary for the development of mature chloroplasts; (*b*) negative regulation of the cGMP-dependent pathway by the calcium dependent pathway; (*c*) negative regulation of the calcium-dependent pathway by the cGMP-dependent pathway.

plant level, which is clearly due to the absence of other phytochromes.

Neuhaus *et al.* (1993) performed initial experiments to show that single cells of *aurea* could respond to microinjected phyA. When dark (D)-grown *aurea* seedlings were transferred to w for 48 h there was no significant chloroplast development nor anthocyanin biosynthesis in hypocotyl cells. However, when phyA purified from oat was microinjected into *aurea* hypocotyl cells, the development of chloroplasts and accumulation of anthocyanin pigments was observed. More significantly, if a plasmid containing a wheat *CAB* promoter fused to GUS (*CAB*-GUS) was co-injected with phyA, expression of the reporter gene could be detected in single cells. Microinjection of the reporter construct alone did not result in expression. Hence exogenous phyA could rescue the *aurea* mutation at the level of gene expression in single cells. This response was not transmitted to other cells indicating cell autonomy in such phytochrome responses.

To test putative signal-transduction components, a range of compounds was microinjected into *aurea* hypocotyl cells (Neuhaus *et al.* 1993). Microinjection of a non-hydrolysable GTP analogue, GTP-γ-S that activates G proteins could fulfill the role of microinjected phyA, causing chloroplast development, anthocyanin biosynthesis and *CAB*-GUS expression. Microinjection of the A subunit of pertussis toxin, which blocks specifically heterotrimeric G proteins (Simon *et al.* 1991), blocked activation of the reporter gene by phyA. Injection of other G protein agonists resulted in similar responses, therefore indicating a role for heterotrimeric G proteins in phytochrome signalling.

Shacklock *et al.* (1992) provided evidence to implicate calcium in physiological responses mediated by phytochrome and the role of calcium in gene expression was investigated using the microinjection system (Neuhaus *et al.* 1993). Microinjection of 0.5–5 μM calcium was sufficient to stimulate chloroplast development and *CAB*-GUS expression. Microinjection of 5000 molecules of calcium-activated calmodulin (CaM) gave similar results. Neither of these treatments resulted in the production of anthocyanin pigments. Furthermore, examination of plastid ultrastructure in microinjected cells showed that whereas phyA and GTP-γ-S stimulated the development of mature chloroplasts, calcium or CaM directed only partial chloroplast development. It was clear therefore, that full chloroplast development required other factors and this indicated a branchpoint in the signalling pathway, downstream of G proteins but upstream of calcium. To study calcium-mediated chloroplast development in more detail, immunofluorescence was used to study the appearance of chloroplast proteins following microinjection. These results showed that calcium was not only able to direct the synthesis of nuclear-encoded chloroplast proteins, but also those encoded by the chloroplast itself. None of the proteins from photosystem I were detected in immunofluorescence assays indicating that further signals were required to direct photosystem I synthesis and assembly.

Whereas G protein activation could stimulate full chloroplast development and anthocyanin biosynthesis, calcium could not promote anthocyanin accumulation and was only able to induce immature chloroplasts that lacked photosystem I and cytochrome b_6f. Bowler *et al.* (1994*a*) investigated the role of further putative signalling compounds involved in the stimulation of full chloroplast development and anthocyanin production. The promoter from the gene encoding chalcone synthase, the enzyme which catalyses the first committed step in anthocyanin biosynthesis, was fused to GUS (*CHS*-GUS) as a marker for the activity of signal-transduction pathways stimulating anthocyanin production. Similarly, the promoter from the ferredoxin $NADP^+$ oxidoreductase gene was fused to GUS (*FNR*-GUS) as a marker for transcription of genes encoding components of photosystem I. Neither *CHS*-GUS nor *FNR*-GUS expression was activated by calcium or calmodulin, although coinjection with phyA or GTP-γ-S resulted in expression of both reporter genes. Microinjection of cGMP with *CHS*-GUS resulted in *CHS*-GUS expression but had no effect on *FNR*-GUS or *CAB*-GUS expression. Furthermore, addition of a

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

membrane permeable analogue of cGMP (8-Br-cGMP) resulted in the expression of endogenous *CHS* genes in dark-adapted soybean suspension cultures. In similar experiments, the expression of *CAB*, *FNR* and *RBCS* was unaffected and even decreased. Bowler *et al.* (1994*a*) suggested that phytochrome-mediated induction of *CHS* is mediated by cGMP. Proof that this is indeed the case came from experiments in which microinjection of an inactive analogue of cGMP (Rp-cGMPS) was able to block phyA-activated or GTP-γ-S-activated *CHS* expression in microinjected aurea cells. Further experiments showed that whereas micro-injection of calcium or cGMP alone was insufficient to stimulate *FNR*-GUS expression, injection of both calcium and cGMP together was necessary and sufficient to activate *FNR*-GUS.

These results led Bowler *et al.* (1994*a*) to propose that phytochrome signalling involves the activation of one or more heterotrimeric G proteins and the subsequent participation of three different pathways that are dependent upon calcium and/or cGMP (see figure 1*a*). The calcium-dependent pathway regulates the expression of photosystem II genes such as CAB, and is able to direct partial chloroplast development. Expression of *CHS* and production of anthocyanin pigments is regulated via a cGMP-dependent pathway, and the participation of a third calcium/cGMP-dependent pathway is necessary for *FNR* expression and full chloroplast maturation.

3. REGULATION OF SIGNAL-TRANSDUCTION PATHWAYS

Having established a number of positive regulatory components of the phytochrome signal-transduction pathway, Bowler *et al.* (1994*b*) used similar techniques to investigate the negative regulatory mechanisms which exist within and between the three signalling pathways to modulate signal flux.

After illumination of soybean suspension cultures, there was a rapid increase in *CHS* transcript levels. However, this increase was transient and transcript levels declined within 7 h after the onset of illumination. This pattern of *CHS* expression is similar to that observed in irradiated parsley cell cultures (Bruns *et al.* 1986; Ohl *et al.* 1989), and the synthesis of anthocyanin biosynthetic enzymes follows similar patterns in the germinating seedlings of some species (Mohr & Drumm-Herrel 1983; Brödenfeldt & Mohr 1988; Kubasek *et al.* 1992). This may be a photo-protection mechanism in newly irradiated plants. The transient expression is reminiscent of signal desensitization in animal systems, where continuous exposure to a stimulus results in decreased sensitivity (Koutalos & Yau 1993; Lefkowitz 1993). Indeed, when light-adapted soybean suspension cultures were exposed to higher light intensities, there was a further peak of *CHS* expression which subsequently declined rapidly. This phenomenon is an example of intrapathway regulation within the cGMP-dependent phytochrome signal-transduction pathway. Bowler *et al.* (1994*b*) showed convincingly that induction and desensitization of *CHS* expression result from changes in cGMP concentration. When cell cultures were incubated with a non-hydrolysable cGMP analogue (8-Br-cGMP) *CHS* expression was both increased and prolonged, mRNA levels remaining high almost indefinitely. Further experiments with agonists and antagonists of cGMP supported conclusions that *CHS* induction is the result of cGMP synthesis, and desensitization is the result of cGMP turnover.

When experiments were carried out in light-adapted soybean cultures (Bowler *et al.* 1994*b*), the increases in *CHS* expression mediated by agonists of cGMP were accompanied by decreases in the expression of both *CAB*, which is regulated by the calcium-dependent pathway, and *FNR* which is regulated by the calcium/cGMP-dependent pathway. This finding was confirmed by microinjection experiments in *aurea*. Furthermore, genistein which was shown to be a specific inhibitor of the cGMP-dependent pathway was able to relieve this negative regulation of *CHS* expression. Hence, there appears to be a negative regulation of the calcium-dependent pathway by some positive regulatory component of the cGMP-dependent pathway (see figure 1*c*). Studies using specific inhibitors of the calcium-dependent pathway in soybean, and microinjection experiments using *aurea* showed that increased activity of the calcium-dependent pathway resulted in decreased *CHS* expression and inhibition of the calcium-dependent pathway resulted in increased *CHS* expression. Hence there appears to be negative regulation of the cGMP-dependent pathway by the calcium-dependent pathway (see figure 1*b*). Microinjection of increasing concentrations of CaM into *aurea* cells showed that only at high concentrations of CaM was negative regulation of *CHS*-GUS observed. This implies that high fluxes through the calcium-dependent pathway are required for negative regulation to exert an effect. This data also suggests that the target of negative regulation within the cGMP-dependent pathway must possess a low binding affinity for one or more regulatory components of the calcium-dependent pathway.

The opposing regulatory mechanisms that mediate negative regulation of the calcium-dependent pathway by the cGMP-dependent pathway and vice versa have been termed reciprocal control (Bowler *et al.* 1994*b*) (see figure 1*b*,*c*). There is both intrapathway negative regulation of phytochrome signal-transduction (as shown by the desensitization of *CHS* expression) and also interpathway negative regulation in the form of reciprocal control. It is possible that negative regulation of the calcium-dependent pathway and the calcium/cGMP-dependent pathway by high levels of cGMP may suppress the synthesis of photosynthetic components during periods of insufficient photoprotectant levels, such as the early stages of de-etiolation. Negative regulation of the cGMP-dependent pathway by the calcium-dependent pathway may then suppress anthocyanin production when these pigments are no longer required.

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

4. ISOLATION OF MUTANTS IN PHYTOCHROME SIGNAL-TRANSDUCTION

A number of laboratories have isolated *Arabidopsis* mutants deficient in phytochrome responses. These mutants fall into two broad classes: light-insensitive mutants which display etiolated phenotypes such as elongated hypocotyls in a range of light conditions, and constitutive mutants which display partially photomorphogenic phenotypes in the dark. Mutants isolated on the basis of long hypocotyls in the light are recessive and may be expected to have mutations in either the photoreceptor or some downstream component of the phytochrome signal-transduction pathway. The mutants characterized so far appear to contain mutated photoreceptors: The *phyB* mutants are deficient specifically in phyB-mediated responses and have mutations in the *PHYB* genes (Koorneef *et al.* 1980; Reed *et al.* 1993). A number of *phyA* mutants, defective in phyA-mediated responses specifically have been shown to possess mutations in *PHYA* genes (Nagatani *et al.* 1993; Whitelam *et al.* 1993; Reed *et al.* 1994). *hy1, hy2* and *hy6* are defective in the synthesis of the chromophore or its incorporation into the phytochrome apoprotein (Parks & Quail 1991; Nagatani *et al.* 1993).

The second class of mutants with altered photomorphogenic responses are those which display constitutive photomorphogenesis in the absence of light. The deetiolated (*det*) mutants and constitutive photomorphogenic (*cop*) mutants (reviewed by Chory 1993; Deng 1994) are recessive indicating that the numerous DET and COP gene products normally repress photomorphogenesis in the dark, rather than transducing positive photomorphogenic signals in the light. DET and COP may be negative regulators of a wide range of developmental pathways, integrating signals from various environmental stimuli including light and plant growth regulators (discussed by Bowler & Chua 1994; Millar *et al.* 1994). We now have knowledge of the structure of four of these negative regulators: FUS6 (Castle & Meinke 1994), COP9 (Wei *et al.* 1994), DET1 (Pepper *et al.* 1994) and COP1 (Deng *et al.* 1992; McNellis *et al.* 1994). Further analysis of these negative regulators should illuminate their role in light signal-transduction.

The large number of constitutive mutants which have been isolated suggests that negative regulators are important components of plant developmental responses, including the transduction and integration of light signals. This is also evident from biochemical data indicating that negative regulation via reciprocal control mechanisms is an important factor in phytochrome phototransduction (Bowler *et al.* 1994*a,b*). However, the biochemical approach of Neuhaus *et al.* (1993) and Bowler *et al.* (1994) has established a number of positive regulatory components within the phytochrome signal-transduction pathway. Attempts to isolate mutants in such components (such as light-insensitive mutants) have so far yielded mostly photoreceptor mutants (see above) with the possible exception of *fhy1* and *fhy3* (Whitelam *et al.* 1993; Johnson *et al.* 1994) and *hy5* (Chory 1992). It is clear that alternative screening strategies are necessary to isolate mutants in other components of the signal-transduction pathway.

5. FAR-RED LIGHT AND PHYTOCHROME MUTANTS

To isolate mutants in phytochrome signal-transduction components, we have restricted our mutant screening strategies to far-red light (FR) regimes to identify mutations in phyA-mediated responses specifically. It is well established that in etiolated seedlings, light-labile phyA is the predominant species. Accordingly, when seedlings are illuminated with continuous FR, the light signal is perceived by phyA specifically. This is due to the relative abundance of phyA compared to phyB and other phytochrome species. FR. illumination is sufficient to convert around 3% of all phytochrome molecules to the active Pfr form which is then degraded. The high abundance of phyA ensures that the response of seedlings to continuous FR illumination *ie* hypocotyl growth inhibition is the result of a phyA-mediated FR-HIR (FR-high irradiance response) (see figure 2). A number of phyA-deficient *Arabidopsis* mutants have been isolated and shown to be insensitive to continuous FR specifically. The *phyA-201* mutant (Nagatani *et al.* 1993; Reed *et al.* 1994) and the *phyA-101* mutant (Dehesh *et al.* 1993; Parks & Quail 1993) both lack functional phyA, and display elongated hypocotyls in FR. Similarly the *phyA-1* mutant (Whitelam *et al.* 1993) which also lacks functional phyA displays elongated hypocotyls only in FR, and normal hypocotyl shortening in red light (R) or W. On the other hand the *phyB* mutants (Koorneef *et al.* 1980; Reed *et al.* 1993) which lack functional phyB, display normal hypocotyl shortening in FR but elongated hypocotyls in R. This phenotypic response to continuous R is therefore mediated largely by phyB. Whitelam *et al.* (1993) isolated two further classes of FR-insensitive mutants termed *fhy1* and *fhy3* which displayed elongated hypocotyls specifically in FR and normal inhibition of hypocotyl growth in R or W. Unlike other FR-insensitive mutants, *fhy1* and *fhy3* contain wild-type amounts of *PHYA* mRNA, phyA protein, and phyA is spectrally active. Furthermore, the *fhy1* and *fhy3* loci are unlinked to the *PHYA* locus.

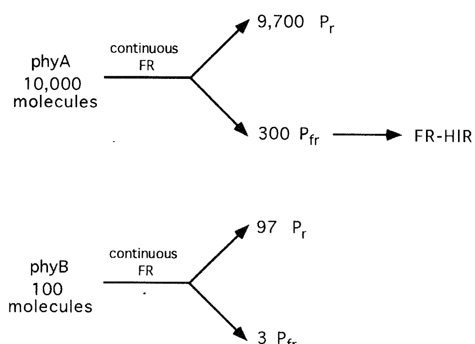


Figure 2. The FR-hir is mediated by phyA. The relative abundance of phyA compared to phyB and other phytochromes in etiolated seedlings ensures that the response of seedlings to continuous FR, which converts a small proportion of phytochrome to the active Pfr form, is mediated by phyA.

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

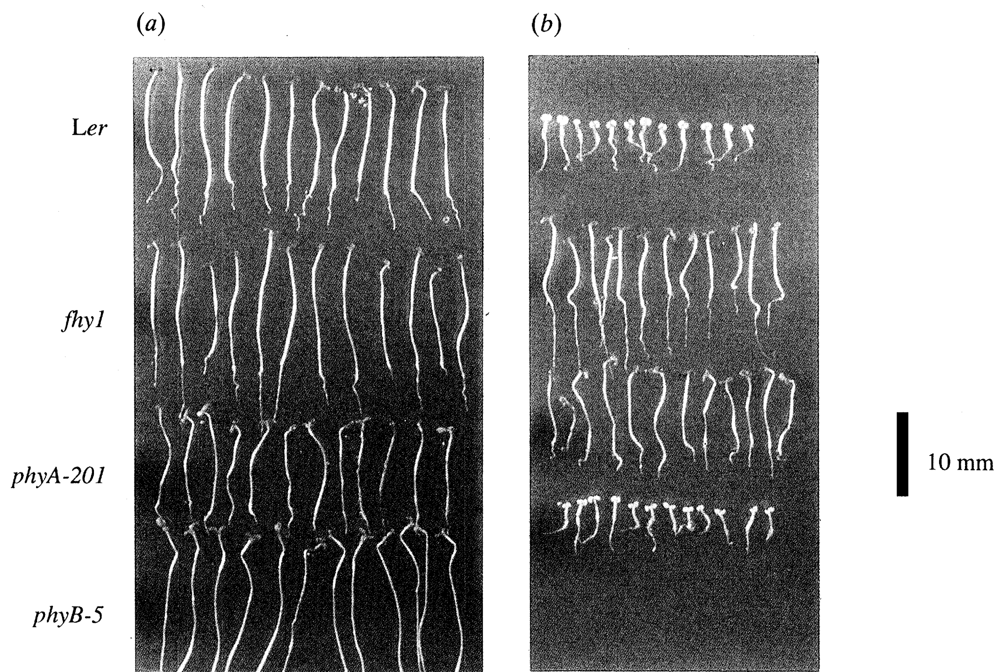


Figure 3. *Arabidopsis* seedlings were germinated and grown at 22 °C for 5 days D, or 1 day D followed by 4 days FR at a fluence rate of 1.5 Wm^{-2}. Seedlings were then transferred to W (2.0 Wm^{-2}) for 48 h at 22 °C. (*a*) seedlings grown in D; (*b*) seedlings grown in FR.

These FR-insensitive mutants are therefore not deficient in the phyA molecule but appear to be very strong candidates for being mutated in downstream components of the phyA signal-transduction pathway.

Our current screening strategy to isolate mutants in phytochrome phototransduction pathways utilizes continuous FR as a means of restricting our screen to defining components of the phyA pathway. This does not exclude mutants blocked in both phyA and phyB signal transduction, and analysis in continuous R will allow us to define branchpoints between the phyA and phyB signal-transduction pathways.

6. A FR BLOCK OF GREENING IN *ARABIDOPSIS* SEEDLINGS

We have established a phyA-mediated response in *Arabidopsis* seedlings, which provides the basis for a simple screening strategy for mutants deficient in positive components of the phyA signal-transduction pathway. When wild-type Landsberg *erecta* (L*er*) seedlings are germinated and grown for 5 days in the dark, the cotyledons become green within 48 h after exposure to continuous W (see figure 3*a*.). The same greening response is observed in the mutants *phyA-201*, *fhy1* and *phyB-5*. However, if seedlings are germinated and grown in the dark for 1 day, followed by 4 days in continuous FR, before exposure to continuous W, the greening response is completely blocked in the wild-type and *phyB-5* (see figure 3*b*). Under the same conditions, *phyA-201* and *fhy1* retain the ability to green in W, displaying insensitivity to the FR block of the greening response. Recently, van Tuinen *et al.* (1995) have established that tomato seedlings grown for 7 days in FR lose their ability to green in W, whereas the phyA-deficient *fri* mutants retain the ability to de-etiolate. In *Arabidopsis* both *phyA-201* and *fhy1* are deficient in phyA-mediated FR perception. Therefore, the FR block of greening which exists in the wild-type and the *phyB-5* mutant is clearly a phyA response. As *phyB-5* responds to FR in the same way as the wild-type, this FR response cannot be mediated by phyB Pr.

When seedlings were germinated and grown for 1 day D followed by 4 days in continuous R, cotyledons were green, even in the *phyB-5* mutant (data not shown). Greening in R may therefore be mediated by a number of photostable phytochromes from the 5

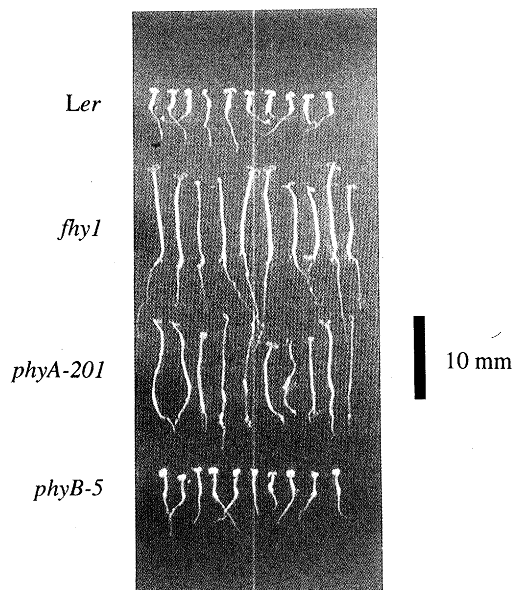


Figure 4. *Arabidopsis* seedlings were germinated and grown at 22 °C for 5 days D, or 1 day D followed by 4 days FR at a fluence rate of 1.5 Wm^{-2}. Seedlings were then transferred to R (3.0–3.5 Wm^{-2}) for a further 4 days at 22 °C.

Downloaded from rstb.royalsocietypublishing.org

Table 1. *Chlorophyll accumulation in* Arabidopsis *seedlings after* FR

(Seedlings were germinated and grown at 22 °C for 5 days D, or 1 day D followed by 4 days FR at a fluence rate of 1.5 Wm^{-2}. Seedlings were then transferred to R (3.0–3.5 Wm^{-2}) for a further 4 days at 22 °C. Chlorophyll a and b measurements were made by homogenizing 50 seedlings in 80% acetone and assaying at 663 nm and 645 nm. Results are shown as mg $seedling^{-1}$.)

	D	D+4 day R	FR	FR+4 day R
L*er*	0.001	0.069	0.001	0.000
fhy1	0.001	0.037	0.004	0.052
phyA-201	0.001	0.041	0.004	0.041
phyB-5	0.001	0.016	0.001	0.000

phyotochrome genes (*PHYA-PHYE*) now known to exist (Sharrock & Quail 1989). We specifically examined whether greening of cotyledons in R (hence phytochrome-mediated greening) is also blocked by growth in FR. The greening response in continuous R required much longer exposures to show clear differences, 4 days of R being sufficient to produce fully green cotyledons. As observed in W, greening of cotyledons in R was also blocked by growth of seedlings in FR (see figure 4). Ler and phyB-5 which had been grown in FR did not accumulate any detectable chlorophyll after 4 days in continuous R (see table 1). Greening of cotyledons was observed in *phyA-201* and *fhy1*, although some seedlings had lost the ability to green. Chlorophyll content in *phyA-201* and *fhy1* grown under FR followed by R was almost identical to that observed in plants grown in D followed by R (see table 1).

The mechanism of such a phyA-mediated FR block of greening in W or R is unclear. It has been established previously that growth of *Arabidopsis* seedlings in the dark for increasing periods of time leads to a gradual loss of ability to green upon transfer to W (Nagatani *et al.* 1993), although these authors asserted that *phyA-201* was less able to deetiolate after growth in the D. FR may therefore simply accelerate this loss of ability to deetiolate which is likely to result from starvation of seedlings. When FR experiments were repeated using seedlings germinated and grown in the presence of sucrose, both L*er* and *phyB-5* retained the ability to green after FR treatment, although the rate of greening was typically slower than that observed in the FR-insensitive *phyA-201* or *fhy1* mutants, or the rate of greening observed in plants grown first in the dark (data not shown). The block in greening may therefore be overcome partly by supplying exogenous sucrose, and may be the result of an acceleration of starvation. L*er* and *phyB-5* grown in the dark for an equal length of time (5 days) in the absence of sucrose retain the ability to green suggesting that FR either accelerates metabolism and depletes energy reserves, or it blocks the liberation of energy reserves from stored carbon, both of which result in premature seedling death. L*er* and *phyB-5* grown first in FR followed by transfer to sucrose-containing growth medium and illumination by W were unable to green, whereas *phyA-201* and

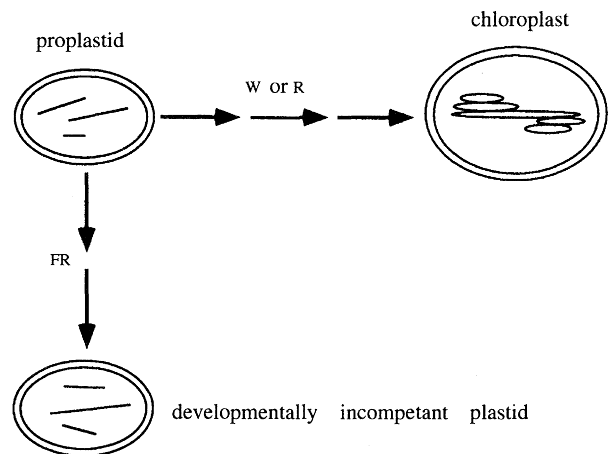


Figure 5. Proposed pathway for FR block of greening. In W or R, normal chloroplast development and maturation occurs. However, FR may mediate partial or abnormal development resulting in plastids which cannot form mature chloroplasts upon illumination by W or R, thus preventing greening.

fhy1 greened faster compared to seedlings which were not transferred to sucrose (S. A. Barnes, unpublished data). Therefore exogenous sucrose cannot rescue seedlings which have already been exposed to FR, and this may suggest that the FR block of greening may comprise elements that are not simple starvation phenomena. Our current hypothesis is that FR causes the abnormal and irreversible development of 'dead end' plastids which may not develop into mature chloroplasts upon illumination by W or R (see figure 5). We are currently investigating the mechanism of the FR block of greening using a variety of approaches.

7. A NOVEL SCREEN FOR PHYA SIGNAL-TRANSDUCTION MUTANTS

Whatever the mechanism underlying the FR block of greening, we have utilized this phenomenon to develop a screening strategy for mutants deficient in the phyA signal-transduction pathway. We have shown that a mutant deficient in functional phyA (*phyA-201*), and a mutant which is potentially blocked in the phyA signal-transduction pathway (*fhy1*) are insensitive to the FR block of greening. By germinating and growing mutagenized M2 seedlings in FR, we are able to select seedlings which retain the ability to green upon transfer to W. By selecting green seedlings from a lawn of non-green plants we are able to screen for mutants which are blocked in the phyA signal-transduction pathway.

Previous morphological screens for mutations in phytochrome perception have concentrated on the isolation of mutants with long hypocotyls in specific light regimes. With the possible exception of *hy5*, *fhy1* and *fhy3* these screens have produced mutants deficient in the phytochrome molecule itself (see above). Although it remains possible that downstream signal-transduction mutants are lethal, the result of long hypocotyl screens suggests that hypocotyl shortening in *Arabidopsis* may depend largely upon the phytochrome molecule itself and that the signal-transduction pathway to gene expression may not be involved (i.e. hypocotyl shortening is via a direct cytoplasmic

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

interaction of the phytochrome molecule with cytoskeletal components). Alternatively, the components of the signal-transduction pathway may be redundant in function leaving the phytochrome molecule as the major controlling determinant of hypocotyl length, and therefore the most likely target of mutant screens.

By using the FR block of greening as a novel screen for signal-transduction mutants we hope to score for a phenomenon which clearly depends upon altered gene expression in the seedling, because any change in metabolism as a result of illumination by FR is highly likely to be the result of altered gene expression. We are currently screening both T-DNA tagged libraries and EMS-mutagenized populations for green seedlings.

Thanks to Ueli Klahre for an enlightening suggestion. S.A.B. is supported by a SERC-NATO Fellowship. R.B.Q. is supported by a CNPq Fellowship from Brazil.

REFERENCES

Bowler, C. & Chua, N. -H. 1994 Emerging themes of plant signal transduction. *Pl. Cell* **6**, 1529–1541.

Bowler, C., Neuhaus, G., Yamagata, H. & Chua, N. -H. 1994*a* Cyclic GMP and Calcium Mediate Phytochrome Phototransduction. *Cell* **77**, 73–81.

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

Brödenfeldt, R. & Mohr, H. 1988 Time courses for phytochrome-induced enzyme levels in phenylpropanoid metabolism (phenylalanine ammonia-lyase, naringenin-chalcone synthase) compared with time courses for phytochrome-mediated end-product accumulation (anthocyanin, quercitin). *Planta* **176**, 383–390.

Bruns, B., Hahlbrock, K. & Schäfer, E. 1986 Fluence dependence of the ultraviolet-light-induced accumulation of chalcone synthase mRNA and effects of blue and far-red light on cultured parsley cells. *Planta* **169**, 393–398.

Castle, L. A. & Meinke, D. W. 1994 A FUSCA gene of *Arabidopsis* encodes a novel protein essential for plant development. *Pl. Cell* **6**, 25–41.

Chory, J. 1992 A genetic model for light-regulated seedling development in *Arabidopsis*. *Development* **115**, 337–354.

Chory, J. 1993 Out of darkness: mutants reveal pathways controlling light-regulated development in plants. *Trends Genet.* **9**, 167–172.

Dehesh, K., Franci, C., Parks, B. M., Seeley, K. A., Short, T. W., Teppermann, J. M. & Quail, P. H. 1993 *Arabidopsis* HY8 locus encodes phytochrome A. *Pl. Cell* **5**, 1081–1088.

Deng, X. -W. 1994 Fresh view of light signal transduction in plants. *Cell* **76**, 423–426.

Deng, X. -W., Matsui, M., Wei, N., Wagner, D., Chu, A. M., Feldmann, K. A. & Quail, P. H. 1992 COP1, an *Arabidopsis* regulatory gene, encodes a protein with both a zinc-binding motif and a Gb homologous domain. *Cell* **71**, 791–801.

Johnson, E., Bradley, M., Harberd, N. P. & Whitelam, G. C. 1994 Photoresponses of light-grown phyA mutants of *Arabidopsis*. *Pl. Physiol.* **105**, 141–149.

Kendrick, R. E. & Kronenberg, G. H. M. 1994 *Photomorphogenesis in plants*. Dordrecht, The Netherlands: Kluwer Academic Press.

Koornneef, M., Rolff, E. & Spruit, C. J. P. 1980 Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) *Heinh. Z. Pflanzenphysiol.* **100**, 147–160.

Koutalos, Y. & Yau, K. -W. 1993 A rich complexity emerges in phototransduction. *Curr. Opin. Neurobiol.* **3**, 513–519.

Kubasek, W. L., Shirley, B. W., McKillop, A., Goodman, H., Briggs, W. & Ausubel, F. M. 1992 Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. *Pl. Cell* **4**, 1229–1236.

Lefkowitz, R. J. 1993 G protein-coupled receptor kinases. *Cell* **74**, 409–412.

McNellis, T. W., VonArnim, A. G. & Deng, X. -W. 1994 Overexpression of *Arabidopsis* COP1 results in partial suppression of light-mediated development: evidence for a light-inactivable repressor of photomorphogenesis. *Pl. Cell* **6**, 1391–1400.

Millar, A. J., McGrath, R. B. & Chua, N. -H. 1994 Phytochrome phototransduction pathways. *A. Rev. Genet.* **28**, 325–349.

Mohr, H. & Drumm-Herrel, H. 1983 Coaction between phytochrome and blue/UV light in anthocyanin synthesis in seedlings. *Physiol. Pl.* **58**, 408–414.

Nagatani, A., Reed, J. W. & Chory, J. 1993 Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome *A. Pl. Physiol.* **102**, 269–277.

Nagy, F., Fluhr, R., Kuhlemeier, C., Kay, S., Boutry, M., Green, P., Poulsen, C. & Chua, N. -H. 1986 Cis-acting elements for selective expression of two photosynthetic genes in transgenic plants. *Phil. Trans. R. Soc. Lond.* B **314**, 493–500.

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

Ohl, S., Hahlbrock, K. & Schäfer, E. 1989 A stable blue-light-derived signal modulates ultraviolet-light-induced activation of the chalcone-synthase gene in cultured parsley cells. *Planta* **177**, 228–236.

Parks, B. M., Jones, A. M., Adamse, P., Koorneef, M., Kendrick, R. E. & Quail, P. H. 1987 The *aurea* mutant of tomato is deficient in spectrophotometrically and immunochemically detectable phytochrome. *Pl. molec. Biol.* **9**, 97–107.

Parks, B. M. & Quail, P. H. 1991 Phytochrome-deficient hy1 & hy2 long hypocotyl mutants of *Arabidopsis* are defective in phytochrome chromophore biosynthesis. *Pl. Cell* **3**, 1177–1186.

Parks, B. M. & Quail, P. H. 1993 hy8, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. *Pl. Cell* **5**, 39–48.

Pepper, A., Delaney, T., Washburn, T., Poole, D. & Chory, J. 1994 DET1, a negative regulator of light-mediated development and gene expression in *Arabidopsis*, encodes a novel nuclear-localized protein. *Cell* **78**, 109–116.

Reed, J. W., Nagatani, A., Elich, T. D., Fagan, M. & Chory, J. 1994 Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. *Pl. Physiol.* **104**, 1139–1149.

Reed, J. W., Nagpal, P., Poole, D. S., Furuya, M. & Chory, J. 1993 Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. *Pl. Cell* **5**, 147–157.

Shacklock, P. S., Read, N. D. & Trewavas, A. J. 1992 Cytosolic free calcium mediates red light-induced photomorphogenesis. *Nature, Lond.* **358**, 753–755.

Sharma, R., Lopez-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. *Pl. J.* **4**, 1035–1042.

Sharrock, R. A., Parks, B. M., Koornneef, M. & Quail, P. H.

BIOLOGICAL SCIENCES THE ROYAL SOCIETY PHILOSOPHICAL TRANSACTIONS OF

BIOLOGICAL SCIENCES THE ROYAL SOCIETY PHILOSOPHICAL TRANSACTIONS OF

Downloaded from rstb.royalsocietypublishing.org

1988 Molecular analysis of the phytochrome deficiency in an aurea mutant of tomato. *Molec. gen. Genet.* **213**, 9–14.

Sharrock, R. A. & Quail, P. H. 1989 Novel phytochrome sequences in *Arabidopsis* thaliana: structure, evolution, and differential expression of a plant regulatory photoreceptor family. *Genes Devl* **3**, 1745–1757.

Simon, M. I., Strathmann, M. P. & Gautum, N. 1991 Diversity of G proteins in signal transduction. *Science, Wash.* **252**, 802–808.

van Tuinen, A., Kerckhoffs, L. H. J., Nagatani, A., Kendrick, R. E. & Koorneef, M. 1995 Far-red light-insensitive, phytochrome A-deficient mutants of tomato. *Molec. gen. Genet.* **246**, 133–141.

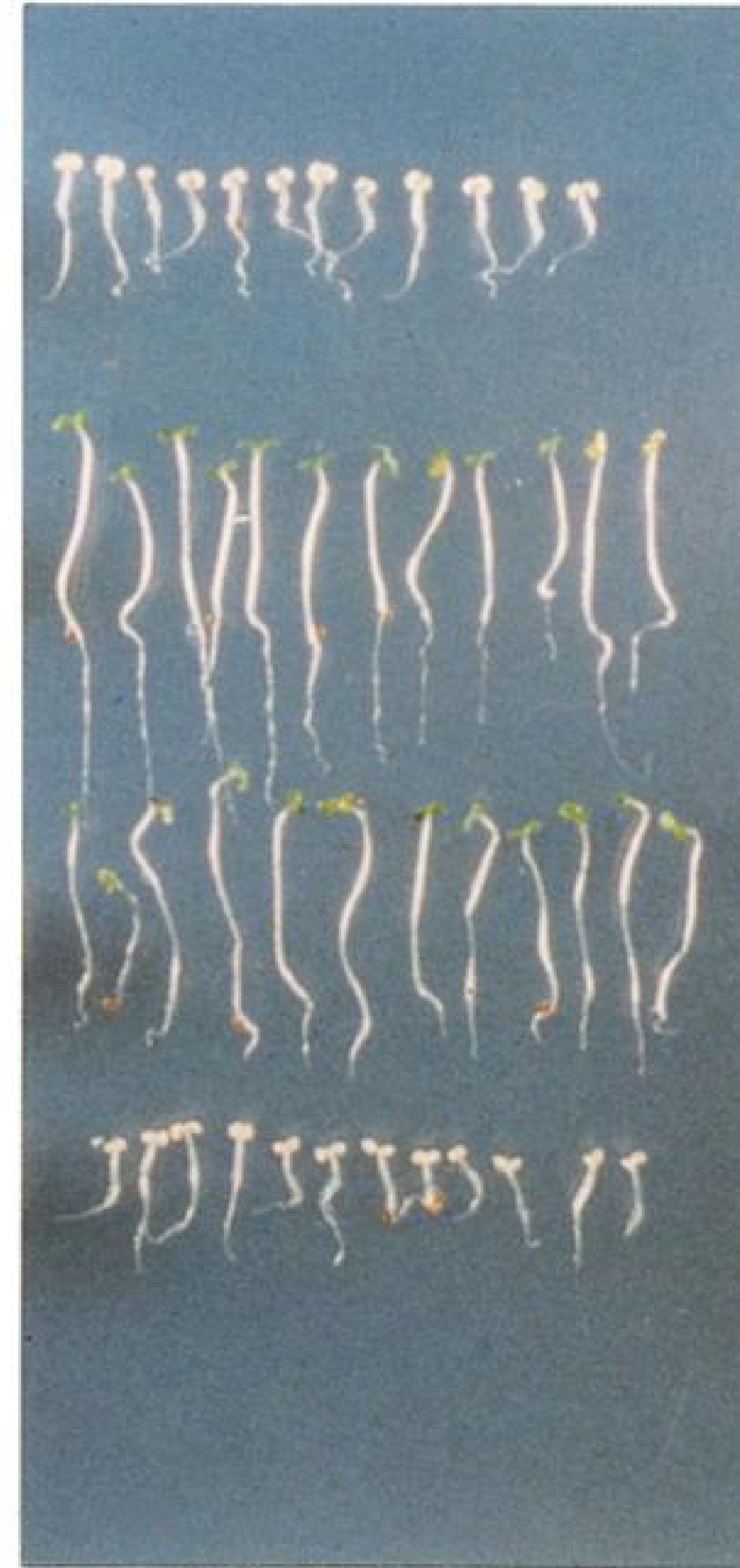
Wei, N., Chamovitz, D. A. & Deng, X. -W. 1994 *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. *Cell* **78**, 117–124.

Whitelam, G. C., Johnson, E., Peng, J., Carol, P., Anderson, M. L., Cowl, J. S. & Harberd, N. P. 1993 Phytochrome A null mutants of *Arabidopsis* display a wild-type phenotype in white light. *Pl. Cell* **5**, 757–768.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

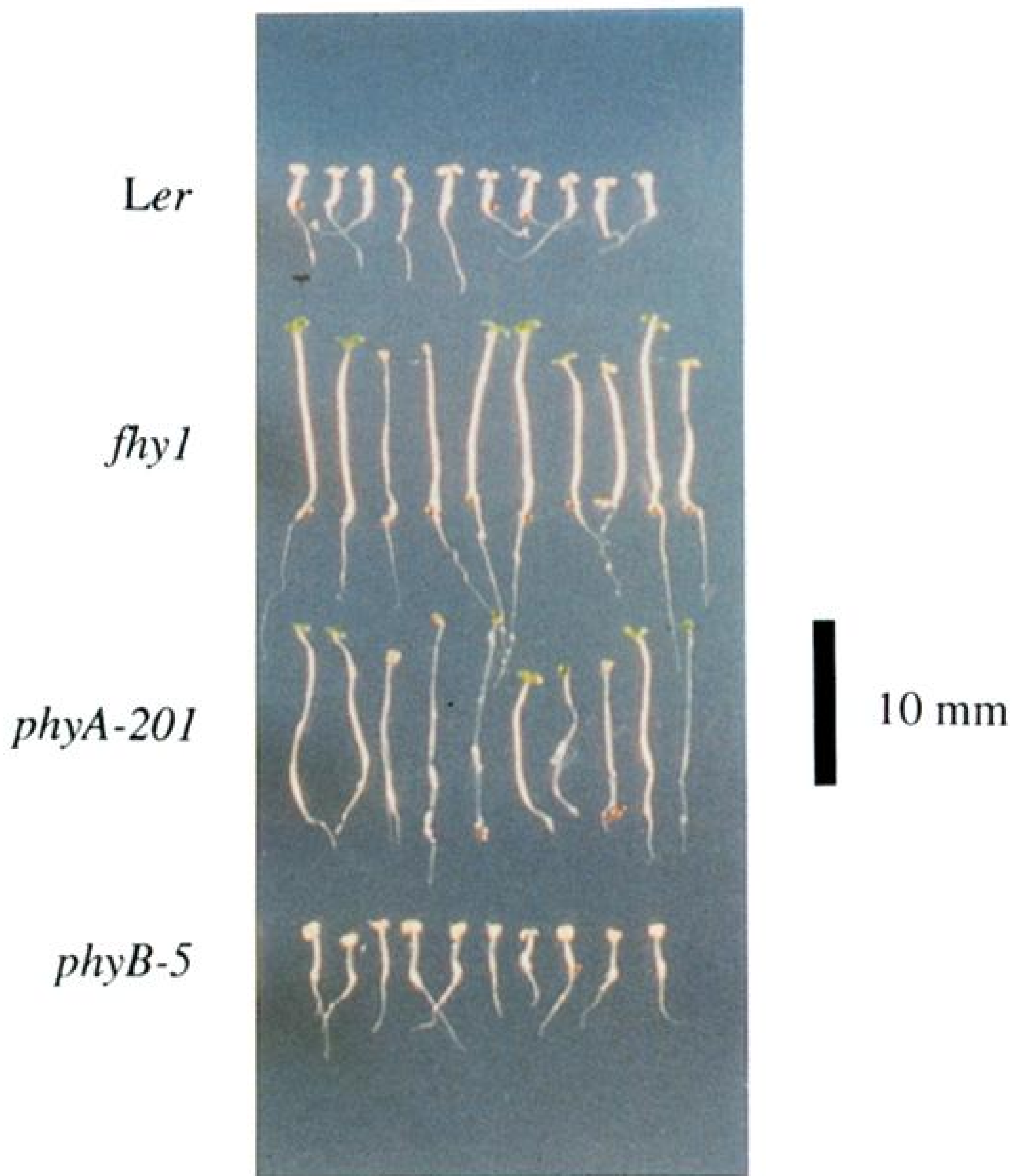
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



gure 3. *Arabidopsis* seedlings were germinated and grown at 22 °C for 5 days D, or 1 day D followed by 4 days FR a fluence rate of 1.5 Wm^{-2}. Seedlings were then transferred to W (2.0 Wm^{-2}) for 48 h at 22 °C. (*a*) seedlings grown D; (*b*) seedlings grown in FR.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



igure 4. *Arabidopsis* seedlings were germinated and grown at 2 °C for 5 days D, or 1 day D followed by 4 days FR at a uence rate of 1.5 Wm^{-2}. Seedlings were then transferred to (3.0–3.5 Wm^{-2}) for a further 4 days at 22 °C.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES