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TECHNIQUES FOR MOLECULAR ANALYSIS

Phenotypic characterization of a photomorphogenic mutant

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Summary

Light is arguably the most important abiotic factor controlling plant growth and development throughout their life cycle. Plants have evolved sophisticated light-sensing mechanisms to monitor fluctuations in light quality, intensity, direction and periodicity (day length). In *Arabidopsis*, three families of photoreceptors have been identified by molecular genetic studies. The UV-A/blue light receptors cryptochromes and the red/far-red receptors phytochromes control an overlapping set of responses including photoperiodic flowering induction and de-etiolation. Phototropins are the primary photoreceptors for a set of specific responses to UV-A/blue light such as phototropism, chloroplast movement and stomatal opening. Mutants affecting a photoreceptor have a characteristic phenotype. It is therefore possible to determine the specific developmental responses and the photoreceptor pathway(s) affected in a mutant by performing an appropriate set of photobiological and genetic experiments. In this paper, we outline the principal and easiest experiments that can be performed to obtain a first indication about the nature of the photobiological defect in a given mutant.

Keywords: phytochrome, cryptochrome, phototropin, light signalling, *Arabidopsis thaliana*.

Introduction

Plants can sense and respond to changes in irradiance, spectral quality, direction and periodicity (day length) of their surrounding light environment (Fankhauser and Chory, 1997). In *Arabidopsis*, seed germination is promoted by light with red being the most efficient waveband, and as little as a few photons can be sufficient to break seed dormancy (Casal and Sanchez, 1998). De-etiolation is initiated when dark-grown seedlings are exposed to light (UV-A/blue, red and far-red light are effective) and involves cessation of rapid hypocotyl growth, unfolding and expansion of the cotyledons, increased pigmentation (chlorophyll, anthocyanin) and organization of the photosynthetic apparatus. After de-etiolation, *Arabidopsis* plants respond to low red to far-red ratios typical of dense canopies by reducing the suppression of petiole elongation, placing the leaves at a more erect position, reducing branching and chlorophyll content and accelerating flowering. Long durations of the daily photoperiod (mainly blue and far-red light) also accelerate flowering (Yanovsky and Kay, 2003). The direction of UVA/blue light induces phototropic responses (Briggs and Christie, 2002). Physiological, biochemical and more recently molecular genetic studies have led to the identification of three families of photoreceptors in higher plants: phytochromes (Quail, 2002b), cryptochromes (Lin, 2002) and phototropins (Briggs and Christie, 2002). Plants also respond to UV-B but the molecular nature of the UV-B photoreceptors is currently unknown.

The phytochromes (phyA–phyE in Arabidopsis) are best known as red far-red photoreceptors, however they do absorb light over the entire visible spectrum and also participate in blue light perception (Casal and Mazzella, 1998; Neff and Chory, 1998). The phytochromes exist in two stable spectral conformations. They are synthesized in the form maximally absorbing red light (Pr). Upon light perception (most effectively red light) Pr is converted to the Pfr form maximally absorbing far-red light (Quail, 2002a). Pfr is most effectively converted back to Pr in response to far-red light. Classic photobiological experiments are consistent with the idea that Pfr is the active form for most but not necessarily all phytochrome-mediated responses (Quail, 2002a). The

functions of the phytochrome family have been particularly well studied in *Arabidopsis* because loss of function mutants in each of the five phytochromes have been identified (Franklin *et al.*, 2003; Monte *et al.*, 2003). Based on these studies one can conclude that phyA and phyB play the most prominent roles and phyD–phyE, and to some extent phyC, have redundant functions with phyB (Franklin *et al.*, 2003; Monte *et al.*, 2003). These results are consistent with the finding that phyA is the only light labile, or type I, phytochrome in *Arabidopsis* and phyB–phyE are all light stable or type II phytochromes (Hirschfeld *et al.*, 1998). phyA can act in two distinct signalling modes, the far-red high irradiance response (FR-HIR) and the very low fluence response (VLFR) to light over the entire visible spectrum (Casal *et al.*, 2000) (see Practical considerations for the definitions of fluence and fluence rate). The FR-HIR allows seedlings to de-etiolate in continuous far-red light (a light quality found under a dense canopy). The VLFR is very important for seed germination (Botto *et al.*, 1996; Shinomura *et al.*, 1996) and presumably acts just as a seedling emerges from the soil and detects light for the first time. Genetic studies indicate that these two pathways are partially distinct (Cerdan *et al.*, 2000; Yanovsky *et al.*, 2002). phyA is also important at later stages of plant development in particular to detect day length extension that accelerates flowering in *Arabidopsis* (Johnson *et al.*, 1994; Yanovsky and Kay, 2002).

phyB is the major photoreceptor mediating de-etiolation in response to red light. However, multiple phytochromes participate in this response (Franklin *et al.*, 2003; Monte *et al.*, 2003; Reed *et al.*, 1994). *phyB* mutants have striking phenotypes throughout development, they are pale, spindly, have long petioles, have increased apical dominance and flower early, particularly in short days (Reed *et al.*, 1993; Whitelam and Devlin, 1997). Similar phenotypes are observed in plants grown in the shade. It was therefore concluded that phyB mutants display a constitutive shade-avoidance phenotype (Whitelam and Devlin, 1997). This phenotype can be explained because phyB in its Pfr form is required to limit growth in several organs (stems, petioles, etc.). In the absence of phyB this growth response is constitutive.

The cryptochromes are UVA/blue light receptors (cry1 and cry2 in *Arabidopsis*) that play key functions during de-etiolation under blue light and photoperiod-controlled induction of flowering. cry1 plays the prevalent role in response to high light intensities and cry2 is most important in response to a low light irradiance (Lin, 2002). This differential sensitivity to irradiance of the two cryptochromes is partially explained by the light-labile nature of cry2 in contrast to cry1, which remains stable in the light (Lin, 2002). The phytochromes also mediate inhibition of hypocotyl growth in blue light, with phyA playing the most prominent role under these conditions (Whitelam *et al.*, 1993). The cryptochromes are very important for blue light-regulated gene expression and anthocyanin accumulation (Ahmad *et al.*, 1995; Lin *et al.*, 1995b; Ma *et al.*, 2001). cry2 has a particularly important function for day length-dependent induction of flowering (Guo *et al.*, 1998; Yanovsky and Kay, 2002). A third cryptochrome (cry3 or cry DASH), divergent from cry1 and cry2, is present in *Arabidopsis* but its function has not been established yet (Kleine *et al.*, 2003).

The phototropins (phot1 and phot2 in *Arabidopsis*) absorb blue light and mediate a number of specific responses including phototropism, stomatal aperture and chloroplast movements (Briggs and Christie, 2002). Phytochromes and cryptochromes modulate this response but the phototropins are the primary photoreceptors (Stowe-Evans *et al.*, 2001; Whippo and Hangarter, 2003). Kinetic analysis of hypocotyl growth has revealed a role for the phototropins in inhibition of hypocotyl growth during the first 30 min in blue light (Folta and Spalding, 2001). The *phot2* mutant is damaged by very high irradiances because of a defect in the chloroplast light avoidance response (Kasahara *et al.*, 2002). Moreover, the *phot1phot2* double mutant displays a leaf curling phenotype that can easily be detected in adult plants (Sakamoto and Briggs, 2002).

Mutants defective for various light responses can be classified in accordance with their phenotype and light sensitivity. A large number of mutants are selectively impaired for de-etiolation in far-red light. As phyA is the only photoreceptor acting under these light conditions they are considered phyA signalling mutants (Quail, 2002b). Similarly mutants selectively impaired for de-etiolation in red light are generally considered phyB signalling components although in this case one has to be more cautious about the interpretation (Hudson, 2000; Quail, 2002b). Additional phytochromes including phyA are required for de-etiolation in red light (Franklin *et al.*, 2003; Monte *et al.*, 2003; Parks and Spalding, 1999; Reed *et al.*, 1994). Moreover, many mutants affecting the circadian clock selectively affect red-light sensitivity (Fankhauser and Staiger, 2002). In addition to the mutants that specifically affect signalling downstream of a single photoreceptor some mutations have a more pleotropic phenotype. *hy5* is the most famous example. The *hy5* mutant has longer hypocotyls than the wild type in all light qualities suggesting that HY5 is necessary for a step that is common to signalling in both the phytochromes and the cryptochromes (Oyama *et al.*, 1997). Mutations affecting the *COP/DET/FUS* class of genes result in de-etiolation in the absence of light (Wei and Deng, 1996). Formally, this indicates that this class of genes code for repressors of photomorphogenesis. Epistasis analysis of photoreceptor mutants and *det*/*cop*/*fus* mutants indicates that they act downstream of the phytochromes and the cryptochromes (Quail, 2002b). This proves the existence of signalling elements common to both classes of photoreceptors acting both positively and negatively.

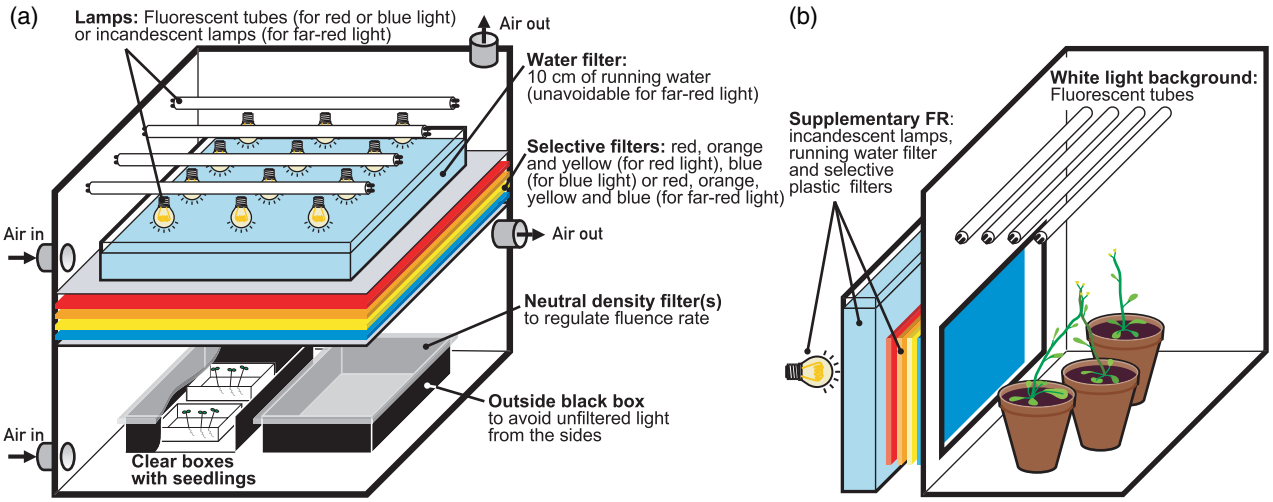

Figure 1. Diagrammatic representation of light sources.
(a) Sources of red, far-red or blue light. These sources can be used to grow etiolated seedlings under continuous (or pulsed) light of selected spectral regions and to give EODFR.
(b) Experimental setting to grow plants under white light plus supplementary far-red light (low red to far-red ratio) simulating neighbour plants. Controls are grown without supplementary far-red light and intermediate red/far-red ratios can be achieved by varying the fluence rate of far-red light.

In this paper, we describe a number of relatively simple experiments that can be performed in order to determine if a mutant has a specific phenotype suggesting a function in light-mediated development. By comparing the phenotypes of such a mutant with those of well-characterized photoreceptor mutants, these experiments should also give some indication about the nature of the signalling branch that is affected.

De-etiolation experiments

The morphological changes that take place during de-etiolation are easy to score and are highly informative of the action of different photoreceptors. De-etiolation experiments are therefore the best starting point.

Basic experimental set-up

Growth chambers with stable temperature are needed to perform de-etiolation experiments with red, far-red and blue light treatments. Most researchers do those experiments under constant light conditions at 20–22°C. Two main strategies can be employed to obtain these wavebands: filtering broad spectrum light with appropriate filters (Figure 1a) or using light emitting diodes (LED) with a known spectral output. Ideally, such a growth chamber should be in a dark room so that when the incubator has to be opened the light in the room does not alter the experiment. A curtain covering the door of the room is useful to minimize exposure of seedlings to light streaming in because of unexpected visitors. In addition to different light qualities irradiance is important. A proper characterization often requires a fluence rate–response curve where hypocotyl growth for instance is determined under a broad range of fluence rates. Neutral density filters can be used to obtain a wide range of irradiances in a single experiment (see Figure 1 for a typical setting and practical considerations for further details). Dark-control seedlings must always be included to assess the actual response to light. The boxes or Petri dishes containing these seedlings can be wrapped in thick black plastic and aluminium foil, either in the light cabinets (this avoids chamber-to-chamber differences in temperature) or in a separate cabinet without light sources. A higher degree of sophistication can be achieved by using protocols with repeated light pulses. This requires a timer or a combination of two timers, the first one sets the frequency of the pulses, governing a second timer that sets the duration of the pulse.

Preparing your seeds

The methods and experimental designs we recommend apply to *Arabidopsis*. The choice of growth medium is an important consideration that will have a major impact on the results. We recommend avoiding the use of sucrose in the growth media. Sucrose and light have a complicated relationship. The most commonly used media is half strength MS with 0.8% phytagar (see Practical considerations). Water agar can also be used but seedlings should not be grown too long on water agar before the phenotypes are evaluated. Water agar certainly has financial and practical advantages (sterilization of the seeds is not critical). For special applications sucrose (e.g. 1.5%) has to be used. In particular, anthocyanin accumulation occurs under certain light conditions only if sucrose is added to the medium. Seeds should be plated at regular intervals to avoid mutual interference with the light field.

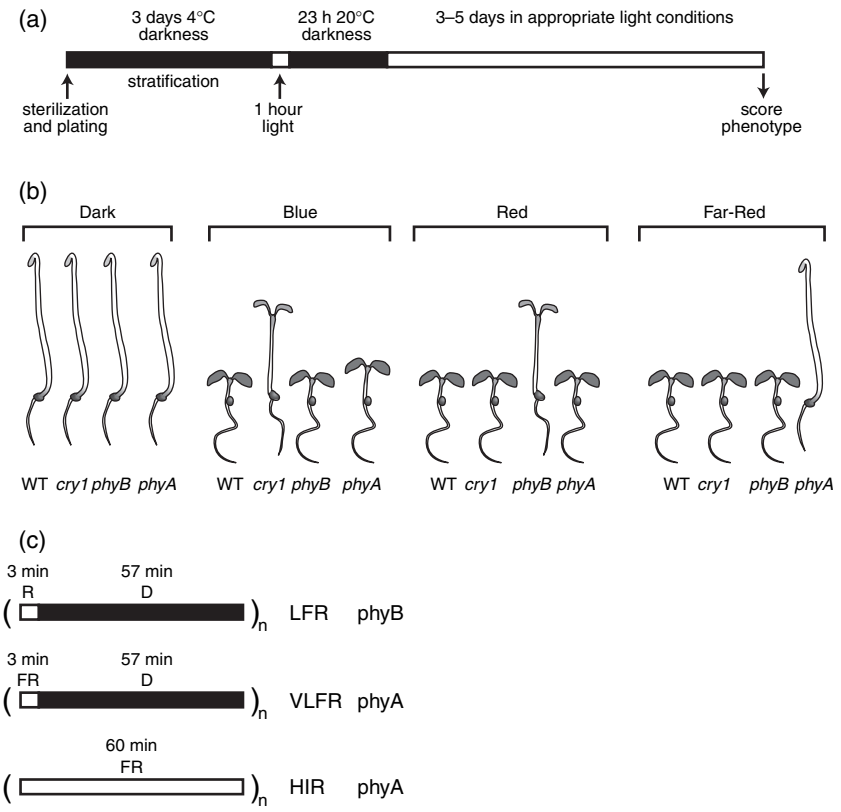

Figure 2. Classic de-etiolation experiments.
(a) Schematic representation of the experimental plan. Sterilized seeds are plated and kept in the dark for 3 days at 4°C (stratification). Germination is induced by a 1 h light treatment and the plates are returned into the darkness at 20–22°C for 23 h. Finally the plates are placed into appropriate light conditions (monochromatic blue, red or far-red light) for 3–5 days before the phenotypes are scored.
(b) Schematic representation of the phenotypes observed for photoreceptor mutants having striking phenotypes in blue (*cry1*), red (*phyB*) or far-red (*phyA*). Note that the *phyA* mutant is 'blind' to far-red light whereas *cry1* and *phyB* only show diminished sensitivity to blue and red light, respectively.
(c) Schematic representation of specialized light regimes allowing the characterization of the phyB LFR, the phyA VLFR and the phyA HIR. These light treatments are typically given for 3 days before phenotypic analysis.

Synchronized germination is extremely important. As germination depends on the age of the seeds and the life of the mother plant we recommend comparing seed batches from mother plants that grew together. To improve synchronous seed germination, sterilized seeds are plated and left in the dark at 4°C for 3–5 days (stratification), then a saturating red-light treatment (e.g. 15 min, 30 μmol m^{-2} sec^{-1}) is administered and the seeds are returned into darkness (22°C) for 24 h before they are moved into the light conditions for a given experiment (Figure 2a). Longer light exposures (1–8 h) can improve germination because light compensates the reversion of Pfr to Pr that can occur in darkness. As fluorescent tubes produce very little far-red, and blue light has little effect on the germination of *Arabidopsis* seeds, their emission can be used instead of red light.

Getting started with hypocotyl length measurements

The easiest phenotype to score is hypocotyl length. We recommend starting with white, blue, red and far-red light. Under fluorescent white light blue and red light, specific phenotypes are normally also visible but far-red light-specific mutants do not have a phenotype because of poor far-red light emission (red/far-red ratio higher than 3). The major photoreceptors sensitive to these light qualities are well characterized and can be used as appropriate controls (Figure 2b). It is also important to properly choose the irradiance. Do not use very high irradiances that completely saturate the hypocotyl growth response because this will prevent you from observing subtle hyposensitive mutants (redundant pathways compensate the defect) and hypersensitive mutants. Blue and far-red light are much more effective than red light to inhibit hypocotyl growth in *Arabidopsis*. As a starting point, we would recommend using about 5 μmol m^{-2} sec^{-1} of blue and far-red and about 20 μmol m^{-2} sec^{-1} of red light. If your wild-type seedlings have 1–2 mm hypocotyl length after one or more of the various light conditions, the irradiance is too strong; rather aim for a 3–4 mm wild type. In most cases, after this initial experiment you probably have to perform a fluence rate–response curve to better characterize your mutant.

In addition to experiments performed in continuous light, pulsed-light treatments are also very informative. As in *Arabidopsis* phyB is the major photoreceptor mediating the red/far-red reversible LFR for de-etiolation an hourly 3-min red light pulse regime is often used as each of these pulses can be followed by a far-red light pulse to test for reversibility. phyA acts in two signalling modes, the VLFR and the HIR (Casal *et al.*, 2000). VLFR were originally defined as the responses induced by a single pulse of very-low-fluence red light (10^{-4}–10^{-1} μmol m^{-2}) or far-red light (which even at higher fluences establishes little Pfr). For the sake of simplicity, the name VLFR can be extended to those

responses that require these pulses to be repeated (e.g. hourly) as for hypocotyl-growth inhibition. Appropriate light treatments can distinguish between the VLFR and the HIR. This is often useful as certain mutants selectively impair one or the other phyA signalling branch. A VLFR treatment is achieved with hourly 3-min far-red-light pulses. An HIR condition is achieved with continuous far-red light (Figure 2c) and is calculated as the difference between the effects of continuous and pulsed far-red at equal total fluence (Casal *et al.*, 1998).

In parallel to growth in the different light qualities it is very important to characterize the phenotype of all the genotypes that are studied in the dark. When a mutant has a shorter hypocotyl than the wild type in the light, one must test if this is a *det*/*cop*/*fus* class mutant that also affects hypocotyl elongation in the absence of light. In addition, hypocotyl length in the dark is often a good indication of synchronous germination.

In most cases, seedlings are grown on horizontal plates (Figure 1). After 3–5 days in the chosen light regime, seedlings are transferred onto acetate sheets scanned and measured, for instance, with NIH image (Neff and Chory, 1998) (see Practical considerations). Alternatively, the seedlings can be laid down straight on a ruler and measured to the nearest millimetre. To correct for late germination one can analyse only the tallest seedlings of each box for each genotype (e.g. 10 out of 15 or 20 seeds). If a genotype shows poor germination more seeds may be needed. As the history of the seeds will influence early seedling development it is not uncommon to express hypocotyl length relative to the length of dark-grown seedlings. This value is remarkably stable despite batch-to-batch fluctuations in the length of dark controls. It is however necessary to show the actual hypocotyl length of each genotype in the dark (which is used as the basis for calculations). We also recommend performing the experiments with the various genotypes from different seed batches to ensure that the observed difference is caused by the genotype and not the growth condition of the parent plants.

When planning an experiment one should take into account that the seedlings grown in the same box or Petri dish are subsamples and not independent samples. If you use 15 seeds per box, the average length of the 10 tallest seedlings is one replicate. Statistics should be based on different boxes, never less than three and as much as 20 or 30 (200–300 seedlings), depending on the precision required to characterize a given effect. After a little practice, it will be found that the main source of error is seedling variability and not the measurement itself. Deviations with respect to true values caused by imprecise measurements are randomly distributed and cancel each other (mean = 0). If you measure the same box several times you will end up with very similar average values. Thus, it is undoubtedly better to devote time to more replicates than to the measurement of each seedling. It is often essential to make measurements blind – in the absence of knowledge of the treatment administered or genotype – especially when the response is small. This procedure avoids bias.

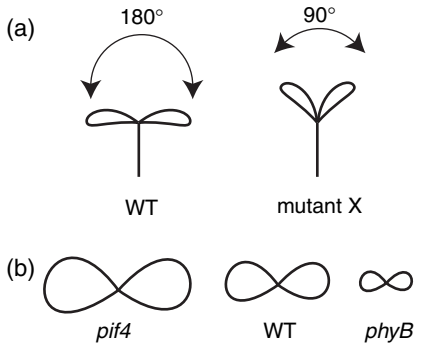

Figure 3. Cotyledon phenotypes of wild type and mutant *Arabidopsis* seedlings.
(a) Mutants that are hyposensitive to light show a reduced opening of the cotyledons that can be measured as the angle between the two cotyledons.
(b) Mutants that are hypersensitive to light (e.g. *pif4*) or hyposensitive to light (e.g. *phyB*) have larger or smaller cotyledons than the wild type (WT) respectively. Similarly to the hypocotyl length phenotype, this phenotype can be specific for a particular wavelength (i.e. *phyB* and *pif4* mutants display this phenotype specifically in red light).

Cotyledon size measurements

Although most people start with hypocotyl length because it is the easiest phenotype to score, light has numerous other effects during de-etiolation and several other informative phenotypic tests can easily be performed. Cotyledon opening and expansion can be measured similarly to hypocotyl length (same growth conditions). The size of the cotyledons can easily be measured from scanned seedlings using NIH image for instance (Figure 3). The cotyledon opening angle is a bit more tricky because one has to ensure that this angle is not perturbed upon seedling transfer to the acetate sheet. To avoid seedling squashing (and angle alteration) we recommend using a protractor with the lines indicating angles extended to the origin rather than a flat-bed scanner. It is difficult to resolve a difference of 10° in cotyledon angle but the response goes from 0 to 180° (or more if you take the tip of the cotyledon), making this error relatively small. In *Arabidopsis*, cotyledon opening is more sensitive to light than hypocotyl growth inhibition. Therefore, this measurement provides a very useful complement, particularly for the analysis of the response to very low light or a pulsed light regime.

Light modulation of gravitropism

In the dark, hypocotyls grow vertically (i.e. away from the gravity source). In contrast, red and far-red light inhibit

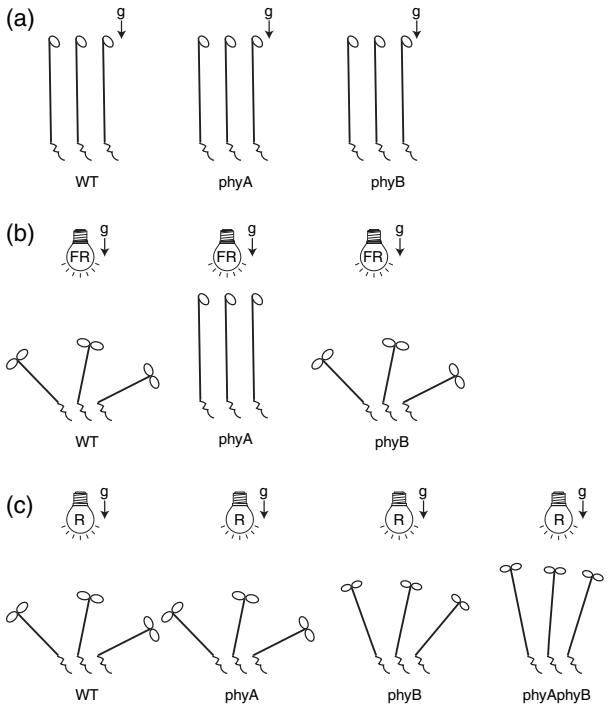

Figure 4. Red and far-red light inhibit gravitropism in *Arabidopsis* seedlings.
(a) Dark grown seedlings show negative gravitropism, they grow against the gravity vector.
(b) Far red light inhibits gravitropism resulting in hypocotyl growth in a random orientation, the *phyA* mutant still growth vertically under these conditions.
(c) Red light inhibits gravitropism resulting in hypocotyl growth in a random orientation, the *phyB* mutant has a reduced response and a *phyAphyB* double mutant growth more vertically than the *phyB* single mutant.

gravitropism resulting in a random growth pattern. This inhibition of gravitropism is a phytochrome response because phytochrome mutants partially restore gravitropism in red light and *phyA* mutants completely restore it in far-red light (Figure 4) (Hangarter, 1997; Poppe *et al.*, 1996; Robson and Smith, 1996). In blue light, the seedlings grow vertically. This may be due either to phototropism towards the blue light source that is present above the seedlings or to normal gravitropism (or a combination of both). To quantify this response we recommend growing the seedlings on vertical plates in the dark and under a few fluence rates of red and far-red light (Fairchild *et al.*, 2000). The plates can directly be scanned and angles to vertical easily measured.

Chlorophyll accumulation

Phytochromes have two opposite effects on chlorophyll accumulation. One is to reduce the lag necessary for chlorophyll accumulation (Lifshitz *et al.*, 1990). Typically, etiolated seedlings (3 days old) are exposed to a pulse of red or far-red light (e.g. 5 min, 1000 μmol m^{-2}), incubated 4 h in darkness and then transferred to fluorescent white light (100 μmol m^{-2} sec^{-1}) for 5 h. Seedlings exposed to the red or far-red (VLFR) pulse have more chlorophyll than dark controls. This experiment requires preliminary work to optimize the protocol. More simple observations are provided by the reduced chlorophyll accumulation of *phyB* mutants grown under continuous white or red light and of the *cry1* mutant grown under blue light (Neff and Chory, 1998).

The second effect of phytochrome on chlorophyll levels is known as the 'far-red blocking of greening'. When seedlings grown for several days under far-red light (note that in the previous paragraph we discuss a few hours) are transferred to white light they fail to synthesize chlorophyll (Barnes *et al.*, 1996). Light regulation of the *PORA* gene coding for the enzyme catalysing the last step of chlorophyll biosynthesis is at the basis of this phenomenon. Etiolated higher plants accumulate high levels of protochlorophylide that is rapidly converted into chlorophyll upon light perception. The PORA protein also accumulates to high levels in the dark allowing rapid conversion of protochlorophylide into chlorophyll once the plant emerges into the light. Light, including far-red light, downregulates *PORA* expression (Sperling *et al.*, 1997). However, the reduction of protochlorophylide is a light-dependent step that is not activated by far-red light so that seedlings grown in far-red light de-etiolate (short hypocotyls and open expanded cotyledons) but they stay yellow. When such seedlings are transferred into white light they have little PORA left and can not accumulate chlorophyll rapidly enough (Sperling *et al.*, 1997). *phyA* mutants are immune to this effect of far-red light because they basically develop as etiolated seedlings (with high PORA levels) in far-red light. phyA signalling mutants can be tested to see if they are more resistant than the wild type to this far-red killing effect (Barnes *et al.*, 1996).

The simplest way to measure this effect is to grow seedlings in far-red light for 3 days, transfer them to white light for 2 days and then measure chlorophyll accumulation. Like other phyA responses, one can test both for an HIR and a VLFR. When seedlings are transferred from a pulsed far-red light regime into white light they will not die. However, seedlings with increased or decreased VLFR responses will accumulate less or more chlorophyll than the wild type, respectively (Figure 5) (Luccioni *et al.*, 2002). The maximum HIR of hypocotyl growth occurs under far-red sources that contain a small amount of light beneath 700 nm. However, the far-red killing effect requires a source devoid of any red light.

Anthocyanin accumulation

Anthocyanin accumulation is a light-dependent process mediated by the phytochromes and the cryptochromes. Anthocyanin levels are much higher when seedlings are grown on sucrose. Growth on 1/2 MS 1.5% sucrose is

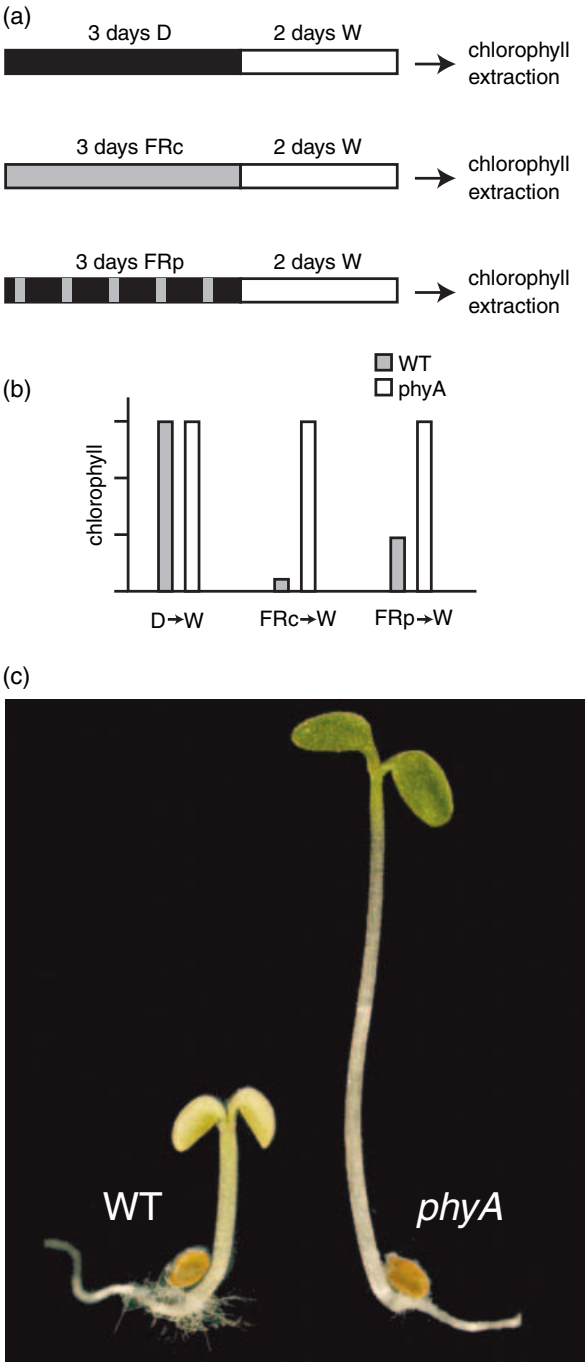

Figure 5. Growth in far-red light inhibits chlorophyll accumulation upon transfer into white light (far-red killing effect).
(a) Schematic representation of the different light treatments. As a control seedlings are grown for 3 days in the dark at 22°C, transferred into white light at 22°C for 2 days followed by chlorophyll extraction. To test the effect of growth in far-red light seedlings are either grown for 3 days in continuous far-red light (FRc) or hourly 3-min pulses of far-red light (FRp). This treatment is followed by 2 days growth in white light and chlorophyll extraction.
(b) Far red light killing measured by chlorophyll accumulation. The expected results for a wild type (WT) and a *phyA* mutant are presented.
(c) Picture of a wild type (WT) and a *phyA* mutant after a far-red light killing experiment. Note the long hypocotyl and green cotyledons of the *phyA* mutant.

necessary to easily detect anthocyanin accumulation under certain light treatments (e.g. pulses of far-red light, see Practical considerations for further details). To assess the specificity of the phenotype, the experiment can be performed in different light colours as for hypocotyl length measurements. The synchrony of germination is very important for this experiment because anthocyanin accumulation is time dependent. There is a peak approximately 3 days after germination followed by a decline in anthocyanin levels (Ahmad *et al.*, 1995). A time course experiment with harvest 2, 3 and 4 days after germination is therefore useful. Anthocyanin accumulation in far-red light is a phyA-dependent process. In blue light cry1, cry2 and phyA are all photoreceptors mediating anthocyanin accumulation but cry1 plays the primary role (Mockler *et al.*, 1999; Neff and Chory, 1998; Poppe *et al.*, 1998). Red light-grown seedlings accumulate much less anthocyanin than seedlings grown in blue or far-red light. However, this measurement is also useful as *phyB* mutants accumulate less anthocyanin than the wild type when grown in red light (Neff and Chory, 1998).

Light-regulated gene expression

Light-regulated gene expression phenotypes are very informative. It is useful to test both rapid and more long-term light responses (Tepperman *et al.*, 2001). Prepare RNA from 4-day-old etiolated seedlings and from etiolated seedlings that were moved for increasing amounts of time into appropriate light conditions. Time points such as 1, 2, 4 and 8 h after light induction are good starting points. To test for photoreceptor specific effects the RNA can be sampled from seedlings moved into blue, red and far-red light. To test for typical phytochrome responses one can use a single red-light pulse (Reed *et al.*, 1994). The etiolated seedlings are treated with a 3-min red-light pulse and returned into darkness. RNA is harvested before and 1, 2, 4 and 8 h after the light pulse. The most commonly used probes are *CAB*, *RBCS*, and *CHS*, but given the large number of light-regulated genes many others can be employed (Tepperman *et al.*, 2001).

Germination

Seed germination is highly sensitive to phytochrome. However, there are several conditions that affect this trait and the experiments have to be carefully designed to avoid confounding effects. The growth condition of the mother plant and the time and environment during storage after harvest can dramatically affect germination. Thus, seeds of all genotypes used in germination tests must be produced in parallel. Exposure of the mother plant to stress or low red/far-red ratios reduces seed germination. During storage of dry seeds at 25°C seed dormancy is reduced and therefore

germination is improved under different light conditions for at least 40 days (Botto *et al.*, 1996). If a genotype affects flowering time we advise repeating the tests with wild-type and mutant seeds of different ages and evaluate whether differences in germination are compensated if the comparison is made at equal time after flowering rather than equal storage time. Germination of *Arabidopsis* seeds is controlled mainly by phyA and phyB but their relative contribution depends on the conditions after the seeds are imbibed and on the light regime (Botto *et al.*, 1996; Shinomura *et al.*, 1996). The seeds (e.g. 25 per sample) can be sown on plain agar-water (0.8%) or on filter paper soaked with the right amount of water (0.06 ml cm^{-2}). Shortly after sowing (1–2 h) and before transfer to full darkness, the seeds can be exposed to a pulse of far-red light to minimize Pfr remaining from seed development. Incubation at low temperature (4–7°C, for 3 days) will reduce dormancy. After this low-temperature incubation and before transfer to darkness at 20–25°C, a pulse of far-red light will promote germination via a VLFR of phyA and a pulse of red light will promote germination via phyA (a pulse of red saturating Pr to Pfr conversion is more than the minimum required to saturate the VLFR) and/or phyB (Botto *et al.*, 1996; Shinomura *et al.*, 1996). The VLFR can also be increased by delaying the far-red light pulse by a day after transfer to 20–25°C (Shinomura *et al.*, 1996). In addition to the major roles played by phyA and phyB, phyE also contributes to the germination response in *Arabidopsis* (Hennig *et al.*, 2002).

Setting a germination experiment requires conditions where the particular response of interest is quantitatively important. If seed dormancy is very strong, a pulse of far-red or even red light may be insufficient to induce germination. Then, increased dry storage and incubation of imbibed seeds at low temperature is recommended. However, there are cases where the seeds show very high germination rates in darkness, or a pulse of far-red causes nearly full germination, leaving no room for a phyB-mediated LFR. Therefore, a period of 1–8 h at 35°C may be necessary to reduce sensitivity and establish the proper starting point. Radicle protrusion is the criterion used to score seed germination. It is convenient to leave the seeds in darkness at 20–25°C before counting germinated seeds (you will find seedlings with long hypocotyls by this time). We recommend the use of probit transformation of the data for statistical analysis (Cone and Kendrick, 1985).

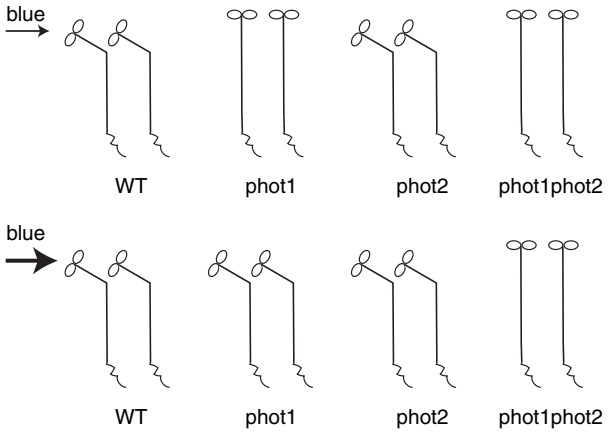

Figure 6. *Arabidopsis* hypocotyls grow towards unilateral blue light. Three days old etiolated seedlings are treated with unilateral blue light for a few hours resulting in hypocotyl bending towards the light source. The phototropins are the primary photoreceptors mediating this light response. Under low blue light a *phot1* mutant is blind and continues to grow strait. Under high blue light only a *phot1phot2* double mutant is blind to this light response.

Phototropism

If the mutant being studied affects phototropism rather than phytochrome- or cryptochrome-mediated signalling, the experiments described so far would not allow detection of a phenotype. Accurate phototropism experiments are quite tricky but it is possible to obtain preliminary data without too much trouble. With the simple phototropic assay that we describe only a rather obvious phenotype can be detected. Seedlings are grown in the dark on vertical plates for 3 days and then illuminated from one side with blue light for 8–10 h (Sakamoto and Briggs, 2002). The phototropic angle can then be measured after scanning the plates (Figure 6). For more careful phototropic experiments we refer the readers to Stowe-Evans *et al.* (2001). The phototropins also control stomatal aperture and chloroplast movements (Briggs and Christie, 2002). Ultrastructure analysis of the chloroplast relocalization response and stomatal aperture assays are beyond the scope of this article (Briggs and Christie, 2002). Such assays have been described elsewhere (Kagawa *et al.*, 2001; Kinoshita *et al.*, 2001; Sakai *et al.*, 2001). *phot2* mutants are defective for the chloroplast avoidance response when plants are exposed to very high irradiances. This phenotype can indirectly be assessed because in the absence of the chloroplast avoidance response leaves are sensitive to very high irradiances (Kasahara *et al.*, 2002). This assay is relatively easy to perform but requires a very strong white light source (more than 1000 μmol m^{-2} sec^{-1}). *phot1phot2* double mutants display a characteristic leaf phenotype that can be observed in adult plants grown in standard conditions (Sakamoto and Briggs, 2002).

Adult phenotypes

The red to far-red ratio of the light and the photoperiod are the two main light signals affecting growth and development of adult *Arabidopsis* plants. In the natural environment, the red/far-red ratio is inversely related to the density of the vegetation canopies and the photoperiod varies with the season.

Low red/far red ratios reduce the suppression of petiole elongation, cause a more erect position of the leaves, accelerate flowering and reduce branching. These effects are collectively called 'shade-avoidance responses' because they increase the competitive ability of plants (Smith, 2000). The red/far-red ratio can be modified without altering light for photosynthesis by adding far-red. The source of far-red (lamps, water and plastic filters as in Figure 1a) can be placed above the white light source. If white light is provided by fluorescent tubes the space equivalent to a tube must be left between one tube and the other to allow far-red to reach the plants. Alternatively, the source can be placed at one side of the plants (Figure 1b). This set-up provides a good simulation of the natural environment, where far-red light reflected on neighbouring plants propagates horizontally (Ballaré *et al.*, 1987). As the source is closer to the plants, the emission does not need to be very strong and this reduces the need to dissipate heat.

Although less representative of the natural neighbour signals, the red/far-red ratio can also be manipulated at the end of the white light period. These classical end-of-day far-red (EODFR) treatments are an easy way to induce shade-avoidance responses. A control set of plants is grown in a 10-h-light 14-h-dark photoperiod (–EODFR). A second set of plants is grown with the same photoperiod but 5 min before they are shifted into the dark they are treated with a saturating pulse of far-red light (+EODFR). The difference between the two treatments is that in the first one plants will start their night with most of their phytochrome in the Pfr conformation, whereas the +EODFR seedlings have most of their phytochrome in the Pr conformation (the far-red light converted phytochrome into Pr). The conformation of the phytochrome will affect, for instance, petiole growth as Pfr will inhibit it but Pr will not. As a consequence, a wild type will have a longer petiole when treated with the EODFR light. These EODFR experiments can also be conducted with young seedlings and hypocotyl length is measured with or without EODFR (Figure 7) (Aukerman *et al.*, 1997; Devlin *et al.*, 1998). *phyB* mutants display a constitutive shade avoidance syndrome. They have long hypocotyls, long petioles and flower early even in the absence of the far-red treatment (Reed *et al.*, 1993). When one compares the hypocotyl length of a *phyB* mutant with and without EODFR treatment there is only a residual response corresponding to the function of the other type II phytochromes (Aukerman *et al.*, 1997; Devlin *et al.*, 1998). More extreme shade avoidance phenotypes can be observed in particular in the *phyBphyE* double mutant (Devlin *et al.*, 1998, 1999; Franklin *et al.*, 2003). The EODFR response of hypocotyl elongation is very informative for a possible function in phyB signalling or signalling downstream of another type II phytochrome (Figure 7).

Arabidopsis is a facultative long-day plant meaning that it will flower more rapidly when grown in long days (16 h light/8 h darkness) than in short days (8 h light/16 h darkness).

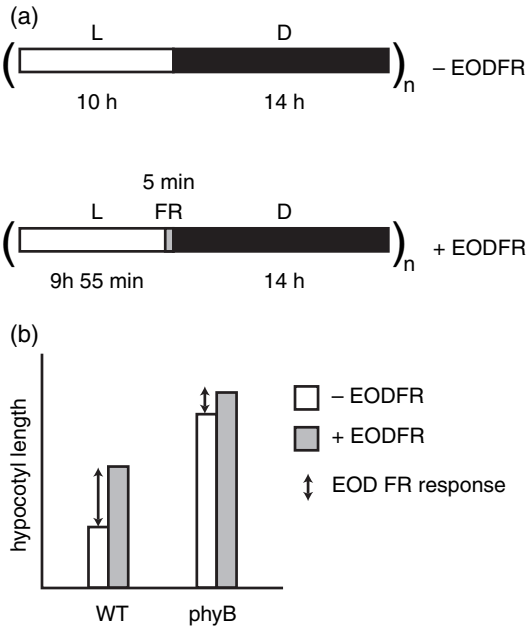

Figure 7. Example of an end of day far-red (EODFR) experiment.
(a) Schematic representation of the experimental plan. After stratification and induction of germination plates are typically left for 2 days in continuous light followed by 4 cycles with (+EODFR) or without (–EODFR) a pulse of far-red light before the night.
(b) Typical phenotype of the EODFR experiment in the wild-type (WT) and a *phyB* mutant. Note that the *phyB* mutant has a very much reduced EODFR response.

Several photoreceptor mutants have quite striking flowering time phenotypes. The *phyB* mutant flowers early under both conditions but the phenotype is more obvious in short days than long days (Blazquez and Weigel, 1999; Reed *et al.*, 1993). It should be noted that this phyB phenotype is particularly sensitive to temperature (Blazquez *et al.*, 2003; Halliday *et al.*, 2003). The *cry*2 mutant flowers normally in short days but is very late in long days (Guo *et al.*, 1998). *phyA* mutants are also somewhat late flowering in long day conditions but this phenotype is more subtle and depends on the quality of the white light. To see the *phyA* phenotype properly day length extensions with low intensities of incandescent light can be performed (Johnson *et al.*, 1994). Flowering time experiments are not easy and the results vary considerably from one lab to the other. This is most probably caused by a large number of uncontrolled variables such as the exact temperature of the growth chambers, the exact light quality, irradiance, the soil etc. However, clear phenotypes such as the one of *cry2* or *phyB* can be observed easily.

Signalling or photoreceptor accumulation mutant?

The phytochromes and the cryptochromes are present in limiting amounts. Careful characterization of the wild type,

heterozygous and homozygous photoreceptor mutant has often revealed that a phenotype can already be observed in the heterozygous state. In addition overexpression studies have shown that a higher dose of either the phytochromes or the cryptochromes results in increased sensitivity to the expected light quality (Boylan and Quail, 1991; Lin *et al.*, 1995b; Wagner *et al.*, 1991). A mutant that affects the accumulation of the phytochromes or the cryptochromes will therefore lead to an altered light sensitivity. Thus, we recommend testing the levels of the relevant photoreceptors by Western blotting. Ideally, this should be performed in the light conditions where the phenotype has been observed. In the case of phyA and of cry2 it is useful to look at the kinetics of light-mediated degradation as both proteins are unstable in specific light conditions (Ahmad *et al.*, 1998; Hirschfeld *et al.*, 1998; Shinomura *et al.*, 1996).

Genetics to the rescue

Numerous photomorphogenic mutants have already been characterized and cloned (Quail, 2002b). Before spending too much time characterizing a mutant that you have discovered it is important to test if you have identified a new allele of an already known gene or if you have really uncovered a new locus required for normal photomorphogenesis. In the case of reverse genetics you can check if the gene that you have disrupted maps close to a known photomorphogenic locus that has not been cloned yet. For any new mutant it is of great importance to ensure that the phenotype you observe is really the result of the disruption of the gene you are interested in. Backcross your mutant and, for insertional mutants, make sure that it is a single insertion event and confirm the data either with a second insertion mutant in the same gene and/or by complementation. If your mutant comes from any random mutagenesis scheme you need to get a rough genetic map position first (Konieczny and Ausubel, 1993). If the new mutant maps in proximity of a known locus it will be important to cross your mutant with the known one to test if you have identified a new complementation group or not.

Photobiological experiments narrow down the set of photoreceptors potentially involved in the pathways affected by your mutant (Table 1). A mutant with a phenotype in far-red light only represents the simplest situation as phyA is the only photoreceptor significantly mediating de-etiolation in far-red light. At the end of the day the only really direct way to ensure that your mutant specifically affects one pathway is double mutant analysis with the relevant photoreceptor mutants. A really careful analysis should therefore include a characterization of the single mutant but also of appropriately chosen double mutants (based on the phenotype of the single mutant). For instance, if the characterization of a new mutant suggests that it is involved in cry1 signalling it is important to make a double mutant with *cry1* but also with *phyA* and *cry2*, the two other photoreceptors mediating de-etiolation in blue light. If the new mutant specifically affects cry1 signalling without affecting other pathways, a *cry1* null mutant should have the same phenotype as a *cry1* double mutant with the new locus (Duek and Fankhauser, 2003). The double mutants with *cry2* and with *phyA* are then expected to behave similarly to the *cry1cry2* and *cry1phyA* double mutants (Duek and Fankhauser, 2003).

Not all mutants affect hypocotyl growth specifically under blue, red or far-red light. Several mutants have phenotypes both in red and far-red light suggesting that they may be involved in phyA and phyB signalling (Choi *et al.*, 1999; Genoud *et al.*, 1998). Other mutants have defects in blue and far-red light (Duek and Fankhauser, 2003; Guo *et al.*, 2001). Such a result does not immediately mean that the mutant is defective for phyA and cryptochrome signalling as a phyA mutant has long hypocotyls in both light conditions (Whitelam *et al.*, 1993). However, appropriate double mutants will determine if the mutant acts downstream of both photoreceptors. In addition some mutants have hypocotyl-growth phenotypes in all light conditions. This suggests that the mutated locus is required for more downstream events. The most famous example is probably the *hy5* mutant (Oyama *et al.*, 1997).

Table 1 Most striking phenotypes of *Arabidopsis* photoreceptor mutants

Genotypes	Phenotypes
cry1	De-etiolation phenotypes in blue light, particularly under high fluence rates
cry2	De-etiolation phenotypes in blue light, particularly under low fluence rates. Late flowering in long days specifically
phyA	Blind to continuous far-red light during de-etiolation (no FR-HIR). No VLFR (germination, etc.). Late flowering in long days
phyB	De-etiolation phenotypes in red light. Reduced end-of-day far-red responses. Constitutive shade-avoidance responses (long petioles). Early flowering particularly in short days
phot1	No phototropic response towards unilateral blue light of low fluence rates
phot2	No chloroplast light avoidance response in the presence of high irradiance

Conclusions

The initial tests to check if a mutant has a photomorphogenic phenotype are relatively simple and do not require any highly specialized equipment. Mutants that are exclusively impaired in de-etiolation in continuous (or pulsed) far-red light are the easiest ones to interpret as phyA is the only

photoreceptor significantly contributing to this light response (Casal *et al.*, 2000). The situation is more complex when dealing with mutants having a defect in blue light perception. cry1, cry2, phyA and to a lesser extent phyB have all been shown to play a role during de-etiolation in blue light (Casal and Mazzella, 1998; Lin, 2002; Neff and Chory, 1998). In addition, it was recently shown that phot1 is the first photoreceptor mediating inhibition of hypocotyl growth when seedlings are transferred into blue light (Folta and Spalding, 2001). However, the transient nature of the phot1 effect makes it difficult to measure its contribution in long-term experiments (hypocotyl growth after several days). De-etiolation defects in red light are also tricky to interpret (Hudson, 2000). phyB is the major photoreceptor contributing to this response but in *Arabidopsis* all five phytochromes are involved (Franklin *et al.*, 2003; Monte *et al.*, 2003). In addition to the direct effect of those photoreceptors alterations of the circadian clock function frequently affect hypocotyl growth in red light specifically. This can incorrectly be interpreted as a specific phyB defect. Given the complex interactions between the light input pathway resetting the circadian clock and the components of the circadian oscillator, teasing light input from circadian oscillator apart is difficult (Fankhauser and Staiger, 2002; Huq *et al.*, 2000; Mas *et al.*, 2003; Reed *et al.*, 2000; Staiger *et al.*, 2003). In this case, we would recommend seeking advice from someone with good photobiological knowledge. This should allow you to more effectively design your experiments and interpret the results.

Practical considerations

Definitions

For a detailed description of the different light measuring units and their meaning please consult Björn and Vogelmann (1994). In general, the most useful information is the photon number on a given surface during a given time. Most commonly expressed as μmol m^{-2} sec^{-1} [where 1 mol equals Avogadro's number or 6.02×10^{23} of photons, and 1 μE (μEinstein) = 1 μmol m^{-2} sec^{-1}]. As an indication, midday sunlight corresponds to approximately 2000 μmol m^{-2} sec^{-1} in the visible range. The advantage of this measuring unit is that photobiological processes depend on the number of photons and when the light field is described in J m^{-2} sec^{-1} (or W m^{-2}) the number of photons depends on the wavelength. The way light is measured has a big influence on the result. If light comes from a single direction as is often the case in incubators with light sources on the ceiling the nature of the light probe does not really influence the result much (a flat light probe is fine). However, if light is diffuse and comes from all directions a spherical probe should be used. When light is measured with a flat probe the term irradiance (expressed in μmol m^{-2} sec^{-1} or W m^{-2}) is appropriate; when the light is measured with a spherical probe the term fluence rate should be used (expressed in μmol m^{-2} sec^{-1} or W m^{-2}). The term intensity is quite commonly used for irradiance. To express a total amount of light measured during a given time (integrated value) the term fluence (expressed in μmol m^{-2} or J m^{-2}) is correct when using a spherical probe and 'time integrated irradiance' when using a flat probe. The term 'fluence' is commonly used while 'time integrated irradiance' is only very rarely employed.

Light sources

The traditional construction of sources is based on the combination of conventional lamps and selective light filters. Red light can be provided by fluorescent tubes in combination with a sandwich of red, orange and yellow acetate or acrylic filters to eliminate short wavebands (Figure 1a). Aluminium foil can be placed on top of the tubes to increase irradiance at plant level. Filter transmittance can be tested in the spectrophotometer (use a clear filter as control). With age, fluorescent tubes emit some far-red and this can be eliminated by interposing a filter containing a copper sulphate solution. Blue light can be obtained by following a similar procedure but using a blue plastic filter (Figure 1a). Blue filters also need careful transmittance evaluation because some are more efficient than others. Far-red light can be provided by incandescent bulbs (spot lamps are useful to avoid wasting light in the wrong direction) in combination with filters that eliminate visible radiation. The latter can be either a combination of red, orange, yellow and blue filters (note that some blue filters cut down much far-red and are not very useful) or dark acetates that eliminate visible light and transmit far-red (Westlake Plastics, Lenni, PA, USA). A 10-cm running-water filter must always be present between the incandescent lamps and the filters (Figure 1a) and is optional for red or blue light sources.

LED sources have numerous advantages: they do not generate much heat, their spectral output does not vary with time, and one can obtain well-defined light qualities. Most researchers use blue light with a peak at 470 nm and a half band width of about 20 nm, red light with a peak at 670 nm and far-red light with a peak at 740 nm. Among other providers Quantum Device sells such ready-to-use LEDs (Quantum Devices Inc., Barneveld, WI, USA, http://www.quantumdev.com/index.html). Moreover, Percival incubators sell a small growth chamber equipped with those LEDs (Percival Scientific, Boone, IA, USA; http://www.percival-scientific.com). The major drawback of this approach is the price. The use of an additional far-red plexiglass filter is required for some experiments (e.g. far-red killing) because about 5% of the light emitted by those far-red LEDs have a wavelength shorter than 700 nm. It is possible to build your own LED panels if you have some help from your electrical shop. Neutral density filters can be used to obtain a wide range of intensities in a single experiment (see Figure 1 for a typical setting). It is useful to carefully select those filters to ensure that they don't distort the spectrum.

A 'safe green light' can be obtained by wrapping a fluorescent light tube with green acetate sheets. It should be noted that no light is completely safe as VLFR are induced by minute amounts of light and in VLFR experiments the seedlings must be handled in complete darkness. However, green light of very low irradiance is the safest (induces the least responses) when working on phytochrome. Very low irradiance red light can be used as 'safe light' when studying cryptochrome and phototropin responses.

Light measurements

In order to measure light irradiance, regular and rather cheap light meters can be used. However, the most common and cheap models only monitor the visible range from 400 to 700 nm. It is therefore impossible to measure far-red irradiance with such light meters. More sophisticated models have to be used in order to obtain data for far-red light (International Light sells such a system equipped

with Filters in order to measure the irradiance at particular wavelength, Newburyport, MA, USA). Ideally, a spectroradiometer measurement would be useful in order to ensure that the spectral output is really what one expects.

Seed sterilization and plating

Seeds are surface sterilized by shaking in 1 ml 70% ethanol with 0.05% Triton X-100 for 5 min followed by 15 min in 100% ethanol. The seeds are then transferred onto sterile filter paper in a hood, dried for a few minutes and sprinkled onto Petri dishes or clear plastic boxes containing 0.8% phytagar (Invitrogen, Carlsbad, CA, USA) and half strength MS salts (Invitrogen). The plates are stored at 4°C in the dark for 3 days before being transferred into the light.

Scanning seedlings for hypocotyl and cotyledon measurements

Essentially performed as described in Neff and Chory (1998). Seedlings sandwiched between two sheets of acetate are scanned in a flatbed scanner at a resolution of 200 dpi. This resolution is sufficient to identify the transition between hypocotyl and root. Digitized seedlings are analysed with NIH Image (http://rsb.info.nih.gov/nih-image/) or Image J (http://rsb.info.nih.gov/ij/). The same images can be used to determine cotyledon size.

Anthocyanin and chlorophyll measurements

Relative anthocyanin levels are determined by collecting 20 seedlings from each of the light treatments/genotype and incubating them overnight in 150 μl of methanol acidified with 1% HCl. Shake the tubes overnight in the dark. The next day, add 100 μl of distilled water and 250 μl of chloroform, vortex and perform a quick spin to separate the anthocyanins from chlorophyll. Total anthocyanins are determined by measuring the A_{530} and A_{657} of the aqueous phase using a spectrophotometer. The relative amount of anthocyanin per seedling is calculated by subtracting the A_{657} from the A_{530}.

Total chlorophyll is determined from samples containing 20 seedlings. Seedlings are extracted by shaking overnight in the dark in 1 ml 80% aceton. Chlorophyll levels are measured spectroscopically and the amount is determined using MacKinney's coefficients (MacKinney, 1941) and the equation: chlorophyll $_{a+b} = 7.15 \times OD_{660nm} + 18.71 \times OD_{647nm}$. When expressed on a per seedling basis, this measurement will also be influenced by cotyledon size. The protocol of Moran and Porath (1980) is often used as well.

RNA extraction and Northern blotting

In experiments with etiolated seedlings the material should be harvested under the minimum irradiance of green light required to handle the samples. It must be borne in mind that even this could induce a VLFR. Etiolated seedlings can be collected by pouring liquid N2 onto the Petri dish, scraping the seedlings off with gloves and collecting them in a cold mortar. They are ground to a fine powder in the mortar and the powder can be kept at −70°C. Approximately 100 mg of seedling powder is then resuspended in 1 ml of Trizol (Invitrogene) and vortexed hard to homogeneity. After 10 min at room temperature, 200 μl of chloroform is added and the samples are vortexed hard for another 15 sec. After 2–3 min at room temperature, the samples are centrifuged at 4°C for 15 min. The aqueous phase is recovered and mixed with 500 μl of isopropanol. After 10 min at room temperature, the solution is centrifuged (in a microfuge) at 4°C for 10 min and the pellet is air-dried. The pellet is then resuspended in 220 μl of DEPC-treated water for 10–15 min. This solution is microfuged for 5 min at 4°C and 200 μl supernatant is precipitated by adding 2.5 volumes EtOH and 1/10 volume of 3 M Na acetate pH 5.5. The RNA is then resuspended in 50 μl of DEPC-treated water at −20°C. Alternatively, 100 mg of seedling powder can be extracted with RNaeasy kits from Qiagen (Valencia, CA, USA). RNA is separated on formaldehyde MOPS gels loaded with 10–15 μg total RNA per lane. The RNA is transferred with 10× SSC onto Hybond N. Probes are generated by random priming. Northern blots are hybridized with Church buffer at 62°C and washed according to the manufacturer's instructions.

Petiole length and flowering time

To determine flowering time we recommend the protocol described by Blazquez and Weigel (1999). Briefly, seeds are planted into pots, stratified for 3 days at 4°C, and transferred into growth rooms at 22°C either in long (16 h light, 8 h dark) or short days (9 h light, 15 h day). The light is provided by a mixture 3:1 cool-white and Gro-Lux fluorescent lights. We strongly recommend that seedlings be not transplanted to avoid stress-induced early flowering. We recommend determining both the number of leaves when the first flower buds appear and the number of days until flowering. We recommend performing the experiments with 18–24 plants of each genotype.

For petiole length determination we recommend the protocol of Devlin *et al.* (1998). Briefly, petiole length is determined from the largest fully grown rosette leaf when the plant has bolted. To determine the end of the petiole we recommend taking the point where the curve goes from concave to convex (beginning of the leaf blade).

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