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Genetic Evidence That the Red-Absorbing Form of Phytochrome B Modulates Gravitropism in *Arabidopsis thaliana*[1]

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Hypocotyls of dark-grown *Arabidopsis* seedlings exhibit strong negative gravitropism, whereas in red light, gravitropism is strongly reduced. Red/far-red light-pulse experiments and analysis of specific phytochrome-deficient mutants indicate that the red-absorbing (Pr) form of phytochrome B regulates normal hypocotyl gravitropism in darkness, and depletion of Pr by photoconversion to the far-red-absorbing form attenuates hypocotyl gravitropism. These studies provide genetic evidence that the Pr form of phytochrome has an active function in plant development.

Phytochrome consists of a family of regulatory photoreceptor chromoproteins that control many aspects of plant growth and development through photoreversible conversions between a Pr form and a Pfr form (Smith and Whitelam, 1990; Quail, 1991). Examples of phytochrome-regulated responses are seed germination, organ growth and development, tropisms, pigment biosynthesis, chloroplast development, the shade-avoidance syndrome, and flowering (Smith, 1982; Kendrick and Kronenberg, 1986; Furuya, 1987; Sage, 1992). The phytochrome gene family consists of several diverse members (Sharrock and Quail, 1989; Quail, 1991). Three of the phytochrome genes in *Arabidopsis* are known to be expressed differently, with *phyA* encoding type I, light-labile phytochrome A and *phyB* and *phyC* encoding type II, light-stable phytochromes B and C, respectively (Quail, 1991). Recent research has shown that these genetically and biochemically distinct phytochromes have discrete functions during plant growth and development (Smith and Whitelam, 1990; Nagatani et al., 1991; Quail, 1991; Parks and Quail, 1993). Conclusions drawn from previous studies have led to the widely accepted view that the Pfr form of phytochrome represents the active form, whereas Pr is thought to be nonfunctional (Kendrick and Kronenberg, 1986; Furuya, 1987; Quail, 1991; Sage, 1992). As shown in this report, however, data obtained from a set of mutants with specific phytochrome deficiencies provides persuasive genetic evidence that the Pr form of phytochrome B is required for normal hypocotyl gravitropism in *Arabidopsis*.

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MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana* (L.) Heynh. ecotypes Landsberg *erecta* and RLD, and mutant strains homozygous for *hy2* (To76), *hy3* (Bo64), *hy3* (8–36) (Koornneef et al., 1980), and *hy8–2* (Parks and Quail, 1993) were used. *hy2* and *hy3* were in the Landsberg *erecta* background, and *hy8–2* was in the RLD background. For surface sterilization and planting on agar medium, seeds were handled as described by Liscum and Hangarter (1993). For biliverdin experiments, the medium contained 0.1 mM biliverdin (Sigma) or methanol at the same concentration found in biliverdin-supplemented medium (Parks and Quail, 1991). Seeds were incubated for 2 to 3 d at 4 ± 1°C on agar medium in Petri plates, then exposed to red light for 30 min to induce uniform germination (Liscum et al., 1992). After the induction of germination, Petri dishes were vertically oriented to allow the seedlings to grow along the surface of the agar and incubated in darkness or in the indicated light conditions at 24 ± 3°C. All manipulations of seedlings were made in dim green light.

Light Sources

Red and far-red light for pulse experiments was obtained by filtering light from two 100-W General Electric soft white incandescent bulbs through filter combinations described previously (Liscum and Hangarter, 1993). Exposure times for each light source and duration of dark periods were computer controlled. Irradiation for 1 min resulted in a fluence of 3300 μmol m^{-2} for each source when measured at 660 ± 20 nm (red) or 730 ± 20 nm (far-red). Red light for continuous irradiations was obtained as described by Liscum and Hangarter (1993) and was given at a fluence rate of 35 μmol m^{-2} s^{-1}.

Green light was obtained by filtering light from one 30-W General Electric incandescent bulb through one layer of Roscolux #86. This light source was considered to be safe because the maximum total fluence the seedlings were exposed to was less than 1 μmol m^{-2}, therefore minimizing the effects of green light on phototropism (Steinitz et al., 1985; Steinitz and Poff, 1986) and on phytochrome phototransformation (Pratt and Briggs, 1966).

Fluence rates at the level of the seedlings were measured with an LI-1800 portable spectroradiometer (Li-Cor, Inc., Lincoln, NE). In experiments using continuous red light, the

$CuSO_4$ solution that was used as part of the light filter was cooled by running tap water through copper tubing submerged in the solution. For experiments with light pulses, cooling of H_2O and $CuSO_4$ solutions was not necessary.

Measurement of Growth Orientation and Statistical Methods

After treatment, the dishes were placed in a photographic enlarger and the seedling images projected ×3.5 were traced. Growth orientation was measured from the tracings in degrees from vertical. Positive angles were assigned to hypocotyls oriented right of the vertical gravity vector, and negative values were assigned to hypocotyls oriented left of the vertical gravity vector.

Because the growth orientation of the different genotypes in darkness and in red light was found to be normally distributed around 0° (vertical) when analyzed for growth orientation, the SD could be used as a measure of the gravitropic response, with a smaller SD corresponding to more vertical growth orientation, or stronger gravitropic response, and a larger SD indicating of randomized growth orientation, or weak gravitropism. When appropriate, the ratio of seedlings falling inside to those outside ± 1 SD of the response of control seedlings were compared by χ^2 analysis (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Stems of most plants exhibit negative gravitropism in darkness so that they grow away from the gravitational vector in a unidirectional manner. Light has been shown to modulate the gravitropic responses of roots and stems in many species through the action of phytochrome (Mohr and Pilcher, 1961; McArthur and Briggs, 1979; Feldman and Briggs, 1987). In most cases, red light stimulates gravity-induced responses (Mohr and Pilcher, 1961; Feldman and Briggs, 1987); however, the gravitropic response of some plants has been reported to be decreased by red light (McArthur and Briggs, 1979).

When wild-type *Arabidopsis* seedlings were grown in continuous (Fig. 1B) or pulsed red light (Table I), their gravitropic response was strikingly attenuated as indicated by an increased degree of randomization of the normal vertical growth pattern exhibited by hypocotyls of dark-grown seedlings (Fig. 1A, Table I). Pulses of far-red light did not result in an increased randomization of the direction of growth (Table I). Moreover, the onset of randomized growth upon exposure to red light was prevented if red light pulses were immediately followed by far-red light pulses (Table I), indicating that this red light-dependent response is mediated by phytochrome. Although previous studies of light effects on gravitropism in *Arabidopsis* failed to show a phytochrome effect (Mirza et al., 1984; Caspar and Pickard, 1989), those studies used white light sources that would not only stimulate the phytochrome-dependent response described here, but also a phototropic response (Steinitz et al., 1985; Steinitz and Poff, 1986), which could interfere with the disorienting effect observed in red light alone.

Three phytochrome-deficient mutant lines of *Arabidopsis*

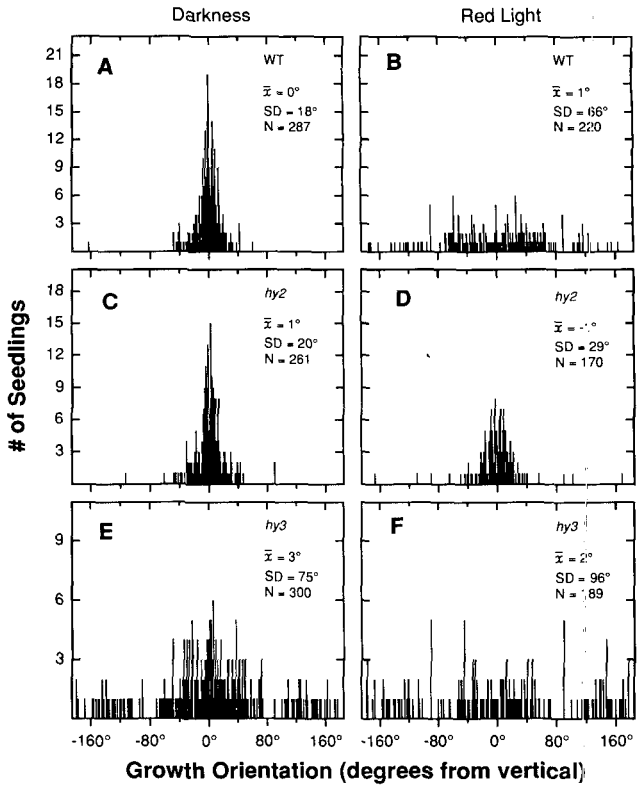

Figure 1. Frequency histograms for hypocotyl growth orientation of dark- and red light-grown wild-type and phytochrome-deficient mutant seedlings. Seedlings and data were handled as described in "Materials and Methods." Orientation of dark-grown wild-type seedlings (A) was centered near vertical, whereas red light-grown wild-type seedlings (B) became disoriented. The *hy2* seedlings were centered near vertical in darkness (C) or red light (D). Seedlings of the phytochrome B-deficient *hy3* mutants were disoriented in both darkness (E) and red light (F). WT, Wild type; x̄, mean; N, population size.

(*hy2*, *hy3*, and *hy8*) were used to investigate further the function of phytochrome in hypocotyl gravitropism. The *hy2* seedlings have wild-type levels of phytochrome A (Chory et al. 1989; Parks et al., 1989) and phytochrome B (Nagatani et al., 1991) apoproteins, but lack photochemically functional phytochrome (Koornneef et al., 1980; Chory et al., 1989; Parks et al., 1989) because of a deficiency in the biosynthesis of phytochromobilin, the phytochrome chromophore (Parks and Quail, 1991). The *hy3* alleles used here, Bo64 and 8–36, were independently isolated and represent near-isogeneic lines (Koornneef et al., 1980). These alleles have been shown to contain different point mutations that result in premature stop codons in the coding region of the *phyB* gene and effectively block synthesis of the phytochrome B apoprotein (Reed et al., 1993). Although both of the *hy3* alleles have been shown to contain wild-type levels of phytochrome A, they do not contain any immunochemically detectable phytochrome B (Nagatani et al., 1991; Somers et al., 1991; Reed et al., 1993). The *hy8* allele used here, *hy8-2*, has been shown to be deficient in immunochemically detectable phytochrome

Table I. *Effect of red and far-red light pulses on hypocotyl growth orientation of wild-type Arabidopsis seedlings*

Every 15 min during a 48-h period, seedlings on vertically oriented agar were exposed to 1 min of red (R), 1 min of far-red (FR), or 1 min of red followed immediately by 1 min of far-red (R/FR). Seedlings sealed against light exposure were included with every experiment as controls (D). After light treatments, the orientation of seedlings was determined as described in Figure 1. The ratio of seedlings falling inside to those outside ± 1 SD of the response of dark-grown, wild-type control seedlings were compared by χ^2 analysis.

Light Condition	Growth Orientation[a]		χ^2	P[b]
	Mean	SD		
	degrees from vertical			
D	−2°	25°		
R	3°	47°	54.32	<0.0005
R/FR	3°	26°	0.46	>0.4
FR	0°	29°	1.33	>0.2

[a] Values represent the pooled mean and SD for a minimum of 200 seedlings from at least two replicate experiments. [b] Control (dark-grown wild type) populations and experimental populations were considered significantly different if $P \leq 0.05$.

A and to have wild-type levels of phytochrome B that appears to function normally (Parks and Quail, 1993).

Regardless of the light treatment, hypocotyls of the phytochromobilin-deficient *hy2* seedlings exhibited negative gravitropism that was similar to the response of dark-grown, wild-type seedlings (Fig. 1, C and D, Table II). A wild-type response to red light (weak negative gravitropism or increased randomization) could be restored in *hy2* seedlings by supplementing the medium with biliverdin (Table III), the immediate precursor to the phytochrome chromophore (Elich et al., 1989; Terry and Lagarias, 1991; Cornejo et al., 1992) that has been shown to restore photochemical functionality to phytochrome in this mutant (Parks and Quail, 1991). These results confirm that phytochrome modulates the normal gravitropic response in wild-type *Arabidopsis* hypocotyls and provides an additional example of a phytochrome-dependent phenotype that can be rescued in *hy2* by application of chromophore precursor (Parks and Quail, 1991).

As demonstrated by tryptic peptide mapping, at least for phytochrome A, the phytochrome apoprotein is synthesized in the Pr form (Parks et al., 1987; Quail, 1991). Thus, because the *hy2* mutant lacks the chromophore necessary for conversion to Pfr, *hy2* plants are expected to have most of their phytochrome apoprotein in the Pr form regardless of light treatment. Although the results obtained with *hy2* demonstrate the involvement of phytochrome in the gravitropic process, they are not sufficient to determine which phytochrome is involved in the red light-induced randomization, or to distinguish whether Pr activates negative gravitropism or if Pfr actively turns it off. To address these points, the effect of red light on the gravitropic response was investigated in the phytochrome A-deficient *hy8* and in phytochrome B-deficient *hy3* mutants.

Red light resulted in significant randomization of hypocotyl growth direction in the *hy8* mutant compared with the *hy8* and wild-type dark controls (Table II). Thus, phytochrome A is not necessary for randomized growth of *Arabidopsis* hypocotyls in red light. However, red light-induced randomization in *hy8* was not as extensive as in the RLD wild type, suggesting that the Pfr form of phytochrome A may play a small role in promoting randomized growth of *Arabidopsis*

Table II. *Effects of dark and red light treatment on the growth orientation of wild-type and phytochrome mutant seedlings*

Seeds were handled as in Figure 1, and the populations were analyzed as described in Table I. D, Darkness; R, red light.

Genotype	Light Condition	Growth Orientation[a]		χ^2	P[b]
		Mean	SD		
		degrees from vertical			
Landsberg	D	0°	18°		
RLD	D	−3°	10°		
hy2 (To76)[c]	D	1°	20°	2.31	>0.1
hy3 (Bo64)[c]	D	3°	75°	165.33	<0.0005
hy3 (8-36)[c]	D	3°	60°	43.10	<0.0005
hy8-2[d]	D	2°	11°	0.63	>0.4
Landsberg[c]	R	1°	66°	201.24	<0.0005
RLD[d]	R	1°	88°	302.11	<0.0005
hy2 (To76)[c]	R	−1°	29°	2.52	>0.1
hy3 (Bo64)[c]	R	2°	96°	192.90	<0.0005
hy8-2[d]	R	−2°	59°	221.07	<0.0005

[a] Values represent the pooled mean and SD for a minimum of 125 seedlings from at least three replicate experiments. [b] Control (dark-grown wild type) populations and experimental populations were considered significantly different if $P \leq 0.05$. [c] The responses of these seedlings were compared with those of dark-grown Landsberg *erecta* wild-type seedlings. [d] The responses of these seedlings were compared with those of dark-grown RLD wild-type seedlings.

Table III. *Restoration of red light-induced randomization of hypocotyl growth orientation by biliverdin in phytochrome chromophore-deficient hy2 seedlings*

Experiments were conducted as described in Figure 1, except that the medium contained 0.1 mM biliverdin or methanol at the same concentration found in biliverdin-supplemented plates (Parks and Quail, 1991). Biliverdin had no effect on growth orientation of wild-type or *hy2* seedlings grown in darkness. The orientation of seedlings in the populations were analyzed as described in Table I. In these experiments, red light-treated wild-type seedlings grown on control medium represented the population that all other treatments were compared with.

Genotype	Biliverdin	Growth Orientation[a]		χ^2	P[b]
		Mean	SD		
		degrees from vertical			
Wild type	−	2°	71°		
Wild type	+	−1°	64°	0.50	>0.6
hy2 (To76)	−	1°	23°	19.48	<0.0005
hy2 (To76)	+	1°	66°	1.69	>0.1

[a] Values represent the pooled mean and SD for a minimum of 50 seedlings from at least three replicate experiments. [b] Control populations and experimental populations were considered significantly different if $P \leq 0.05$.

hypocotyls in red light. It is important to note, however, that the *hy8* mutant was only recently isolated (Parks and Quail, 1993) and may not be isogeneic.

A more striking result was obtained with the phytochrome B-deficient *hy3* mutant. In contrast to dark-grown, wild-type controls, hypocotyl growth in *hy3* seedlings was significantly randomized in darkness (Fig. 1, A and E, Table II). Because two independently isolated *hy3* lines showed randomized hypocotyl growth direction in darkness, this abnormal phenotype is more likely to be a result of the phytochrome B deficiency than a second site mutation. Red light did result in a slightly higher degree of randomization of growth in *hy3* hypocotyls compared with dark-grown *hy3* controls (Fig. 1, E and F), but this may be due to the function of the phytochrome A that is present in the *hy3* mutant.

In addition to demonstrating that phytochrome B is involved in regulating the red light-induced randomization of hypocotyl growth in *Arabidopsis*, the *hy3* mutant shows that the Pfr form of phytochrome B does not actively cause randomization of hypocotyl growth direction because the *hy3* mutant exhibits randomized growth in the absence of both forms of phytochrome B. Moreover, because the *hy2* mutant shows negative gravitropism in the presence of phytochrome B, and because phytochrome B is likely to be in the Pr form in the absence of chromophore (Parks et al., 1987; Quail, 1991), it follows that the Pr form of phytochrome B is involved in regulating negative gravitropism in *Arabidopsis* hypocotyls. Thus, the only model that appears to be consistent with all of the data presented here for wild-type plants and for the different phytochrome-deficient mutants is that the randomization of hypocotyl growth direction in response to red light is due primarily to depletion of the level of the Pr form of phytochrome B through its photoconversion to Pfr.

Other gravity responses have not yet been analyzed in detail in the phytochrome-deficient mutants, but in preliminary experiments light did not appear to affect root gravitropism in *Arabidopsis* (data not shown). However, gravitropic curvature of hypocotyls in response to reorientation from a vertical to a horizontal position showed results that are consistent with the proposed involvement of the Pr form of phytochrome B (data not shown). In particular, dark-grown *hy3* and red light-treated wild-type hypocotyls showed reduced gravitropic response upon reorientation in comparison with dark-grown wild type, whereas *hy2* hypocotyls responded in a similar manner to the dark-grown wild type regardless of red light treatment.

Although there is a vast body of evidence (Kendrick and Kronenberg, 1986; Quail, 1991) that suggests that it is the Pfr form of phytochrome that plays an active role while the Pr form is inactive in phytochrome-dependent responses of a wide range of plant species, several studies have led to the suggestion that both Pfr and Pr function to regulate stem elongation in light-grown plants (Smith, 1981, 1983, 1990). The results presented here, that normal negative gravitropism in *Arabidopsis* hypocotyls occurs when phytochrome B is in the Pr form (as indicated by the *hy2* mutant) and is altered in the absence of phytochrome B (as indicated by the *hy3* mutant) provide compelling evidence that the Pr form of phytochrome B is required for negative gravitropism in *Arabidopsis* hypocotyls. While it remains to be seen if other responses are controlled by the Pr form of the various phytochromes, the demonstration here that distinct biological activities can be associated with each of the two interconvertible forms of phytochrome may lead to a better understanding of how the family of phytochrome photoreceptors controls the wide range of responses that have been observed to be regulated by phytochrome in plants.

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