

Brassinosteroid Mutants Uncover Fine Tuning of Phytochrome Signaling¹

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Phytochromes (phy) A and B provide higher plants the ability to perceive divergent light signals. phyB mediates red/far-red light reversible, low fluence responses (LFR). phyA mediates both very-low-fluence responses (VLFR), which saturate with single or infrequent light pulses of very low fluence, and high irradiance responses (HIR), which require sustained activation with far-red light. We investigated whether VLFR, LFR, and HIR are genetically coregulated. The Arabidopsis *enhanced very-low-fluence response1* mutant, obtained in a novel screening under hourly far-red light pulses, showed enhanced VLFR of hypocotyl growth inhibition, cotyledon unfolding, blocking of greening, and anthocyanin synthesis. However, *eve1* showed reduced LFR and HIR. *eve1* was found allelic to the brassinosteroid biosynthesis mutant *dim/dwarf1*. The analysis of both the brassinosteroid mutant *det2* in the Columbia background (where VLFR are repressed) and the *phyA eve1* double mutant indicates that the negative effect of brassinosteroid mutations on LFR requires phyA signaling in the VLFR mode but not the expression of the VLFR. Under sunlight, hypocotyl growth of *eve1* showed little difference with the wild type but failed to respond to canopy shadelight. We propose that the opposite regulation of VLFR versus LFR and HIR could be part of a context-dependent mechanism of adjustment of sensitivity to light signals.

Light perceived by phytochromes strongly affects growth and development throughout the life cycle of plants. The relevant light signals are widely divergent in different developmental contexts as illustrated by the following examples. First, whereas a brief exposure to light is often enough to promote the germination of weed seeds during soil tillage (Scopel et al., 1991), prolonged exposure to light is required to achieve full seedling de-etiolation. Second, stem growth inhibition is initiated by seedling emergence under high as well as under low red light (R) to far-red light (FR) ratios (Yanovsky et al., 1995; Smith et al., 1997). However, this R/FR ratio-compensated light control of axis growth (i.e. regulation buffered against changes in R/FR) is lost during the de-etiolation process itself and plants become competent to respond to reductions in R/FR ratio caused by vegetation canopies (Holmes et al., 1982). Third, de-etiolation is partially buffered against the different photoperiods that the seedling can face according to the date and place (latitude) of emergence from the soil (Mazzella and Casal, 2001). However, photoper-

iod is a key signal controlling the timing of flowering once the plant has surpassed the juvenile phase of development.

The wide array of light signals that phytochromes can perceive has been conceptualized as three modes of action (for review, see Casal et al., 1998). The very-low fluence response (VLFR) mediated by phytochrome A (phyA) is induced by radiation between 300 and 780 nm (Botto et al., 1996; Shinomura et al., 1996). Brief light exposures are enough (although in some cases these exposures have to be periodically repeated to show a detectable effect; Casal et al., 2000). The low-fluence response (LFR) mediated by phytochrome B (phyB; and to a lesser extent phytochromes D, E, and probably C) is induced by R and not by FR (McCormac et al., 1993; Aukerman et al., 1997; Mazzella et al., 1997; Devlin et al., 1998). Actually, FR is able to revert the Pfr of phyB established by R to physiologically irrelevant levels. This results in the classical R/FR reversibility of LFR. The high-irradiance responses (HIR) mediated by phyA require sustained excitation with FR (Casal et al., 2000). Thus, light control of seed germination in many weeds is dominated by the VLFR component, de-etiolation under dense or open canopies is respectively dominated by the HIR or LFR components, the response to FR back-reflected by neighbors is dominated by the LFR, etc.

Adequate responses to the light environment require the correct hierarchy of these modes of action in each context, but we are relatively ignorant of the mechanisms that regulate such hierarchy. To identify elements of these mechanisms, we designed a protocol to search for mutants with enhanced VLFR dur-

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ing de-etiolation and investigated LFR and HIR in these genetic variants.

RESULTS

Isolation of the *eve1* Mutant

The *eve1* (*enhanced very-low-fluence responses 1*) mutant was identified in a screening of M2 seed of *Arabidopsis* ecotype Landsberg *erecta* by its short hypocotyl and opened cotyledons under hourly pulses of FR (Fig. 1A). The hypocotyl was already shorter than the wild type (WT) in darkness (Fig. 2A) but the VLFR (i.e. the first phase of the response to light pulses providing different Pfr/P) was significantly enhanced in *eve1*. This enhanced VLFR of hypocotyl growth was obvious both when length was expressed relative to the dark controls ($P < 0.001$; Fig. 2B), and when length was expressed in absolute terms (length reduction caused by FR pulses providing a calculated Pfr/P = 10% compared with darkness: WT = 1.2 mm; *eve1* = 3.1 mm; $P < 0.05$; Fig. 2A). The cotyledons of *eve1* seedlings grown in darkness remained fully closed (Fig. 1A). Although the effect was not as dramatic as in the case of hypocotyl growth, the VLFR of cotyledon unfolding was also enhanced in *eve1*. The plateau reached by the VLFR (Pfr/P between 10% and 33%) was significantly higher in *eve1* than in the WT (Fig. 2C, $P < 0.05$).

The F₁ generation of crosses between the WT Landsberg *erecta* and *eve1* was similar to the WT in darkness and under hourly FR pulses (data not shown). Under pulsed FR the F₂ generation showed a 3:1 segregation (22 seedlings with the *eve1* phenotype in 87 F₂ seedlings; $\chi^2 = 3.6 \cdot 10^{-5}$; $P > 0.99$). The adult phenotype of *eve1* showed small rosettes and short stature (Fig. 1B). Flowering time under greenhouse conditions was normal (leaves at flowering \pm SE: WT = 13.1 ± 0.5 ; *eve1* = 13.3 ± 0.9). The short hypocotyl in darkness and the dwarf phenotype of the adult plant cosegregated in F₂ populations. The angle of the cotyledons under pulsed FR was significantly higher in F₂ plants that subsequently showed the *eve1* compared with the WT adult phenotype (cotyledon angle, degrees: WT adult phenotype = 57 ± 8 ; *eve1* adult phenotype = 123 ± 7 ; $P < 0.0005$). Thus, the adult phenotype cosegregates with the enhanced VLFR (as the cotyledons do not unfold in darkness, all the difference under pulsed FR is because of the VLFR). This indicates that all the observed features were caused by the same locus.

eve1 Is Allelic to *dwf1/dim* Mutants

The F₂ of *eve1* Landsberg *erecta* \times WT Columbia was used to map the mutant to the upper arm of chromosome 3, 20.4 cM apart from the marker nga172. The *dwf1/dim* mutants map in the vicinity of this location and also show a dwarf adult phenotype and reduced hypocotyl growth (Takahashi et al.,

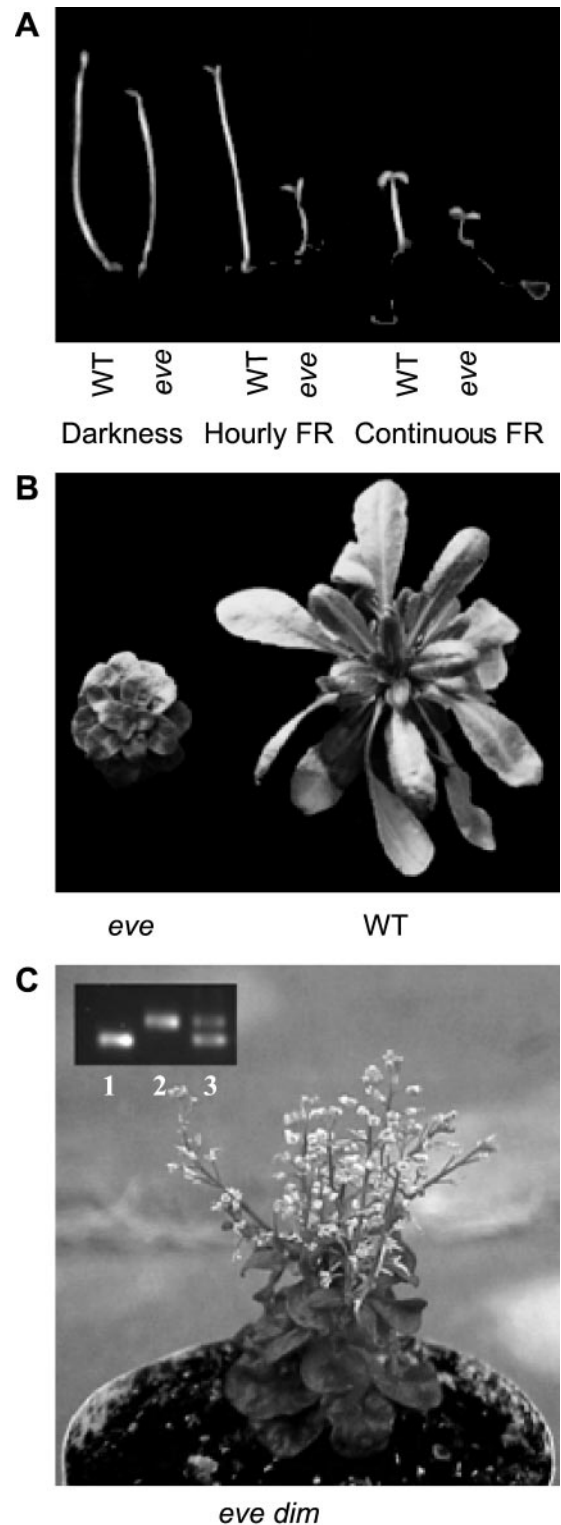


Figure 1. Phenotype of *eve1* seedlings (A) and adult plants (B) and of the F₁ generation between *eve1* and *dim* (C). In C, inset, a PCR marker for the *dim* (*dwf1-2*) allele was used in seedlings homozygous for *eve1* (lane 1) or *dim* (lane 2). The presence of the *dim* allele in lane 3 demonstrates that the seedling in the photograph is product of a successful cross between *eve1* (mother plant) and *dim*.

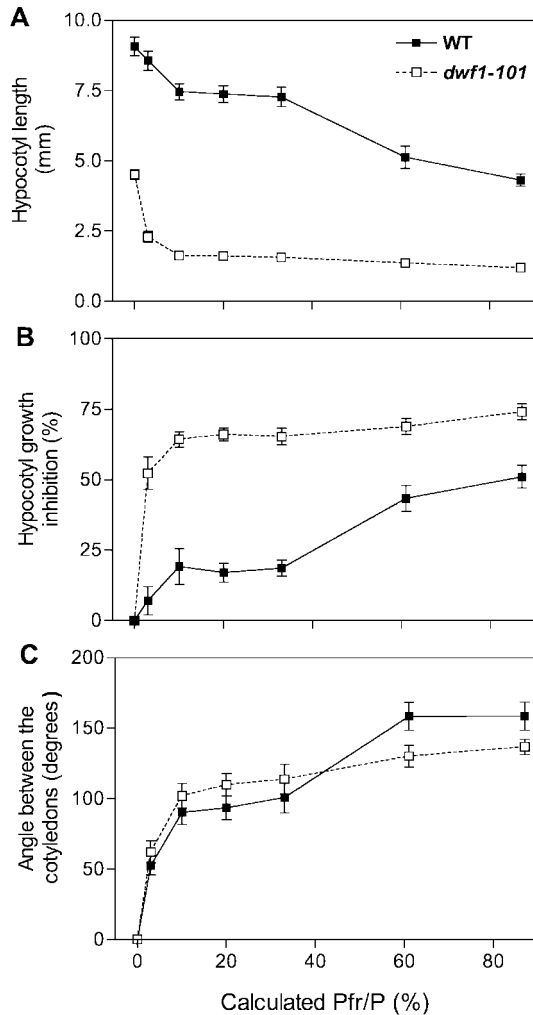


Figure 2. *dwf1-101* (previously designated *eve1*) shows enhanced VLFR and reduced LFR of hypocotyl growth (A and B) and cotyledon unfolding (C). The seedlings were exposed to hourly R/FR pulses predicted to establish the calculated Pfr/P displayed in abscissas. In B, the difference between hypocotyl length in darkness and a given light condition is expressed relative to the length in darkness. Data are means \pm SE of 18 replicate boxes.

1995; Klahre et al., 1998; Choe et al., 1999). The *eve1* mutant failed to complement *dim/dwf1-2* (Fig. 1C), whereas the F₁ generation of crosses between the WT and *eve1* or *dim* showed a WT phenotype. Thus, *eve1* is allelic to *dwf1/dim* and was renamed *dwf1-101*.

Reduced LFR in *dwf1-101*

The slope of the LFR of hypocotyl growth was reduced in *dwf1-101* (percent inhibition/percent calculated Pfr/P, between 33% and 87%: WT = 0.60 ± 0.1 ; *dwf1-101* = 0.16 ± 0.07 ; $P < 0.001$; Fig. 2B). In *dwf1-101*, hypocotyl growth inhibition under continuous FR ($83\% \pm 1\%$) was even stronger than the maximum reached under LFR conditions ($74\% \pm 1\%$; $P < 0.001$), indicating there was room for a significantly stronger hypocotyl response. The *dwf1-101*

mutation elevated the plateau of the VLFR of cotyledon unfolding but decreased the plateau of the LFR ($P < 0.05$, Fig. 2C). Thus, *dwf1-101* showed enhanced VLFR but reduced LFR.

Reduced HIR in *dwf1-101*

One of the distinctive features of the HIR of phyA is its strong fluence-rate dependency (Fig. 3). The largest difference between *dwf1-101* and the WT was observed at the lowest fluence rate of continuous FR tested here and gradually decreased at higher fluence rates. A mutant enhancing HIR should present a steeper fluence rate-response relationship, and a mutant without effects on HIR should produce parallel curves. The reduced slope observed for hypocotyl growth inhibition suggests a negative effect of the *dwf1-101* mutation on HIR. The angle between cotyledons was higher in *dwf1-101* only for the lowest fluence rate tested (degrees, WT = 8 ± 3 ; *dwf1-101* = 42 ± 10 ; $P < 0.01$).

The HIR is the portion of the effect of continuous FR that cannot be mimicked by hourly pulses of the same spectral composition providing the same total fluence. Thus, to measure the HIR we compared several responses in seedlings exposed to pulsed or continuous FR (Fig. 4). The *dwf1-101* mutant showed enhanced VLFR of hypocotyl growth inhibition ($P < 0.005$), cotyledon unfolding ($P < 0.05$), blocking of greening after transfer to white light ($P < 0.005$), and anthocyanin levels ($P < 0.07$). The HIR was significantly reduced in each case ($P < 0.01$), except for blocking of greening ($P > 0.5$). Compared with the WT, anthocyanin levels were reduced in *dwf1-101* seedlings grown under continuous FR ($P < 0.05$). Thus, *dwf1-101* showed enhanced VLFR but reduced HIR.

Reduced LFR and HIR in *det2*

To investigate whether the enhanced VLFR is a feature common to other mutants affecting brassinos-

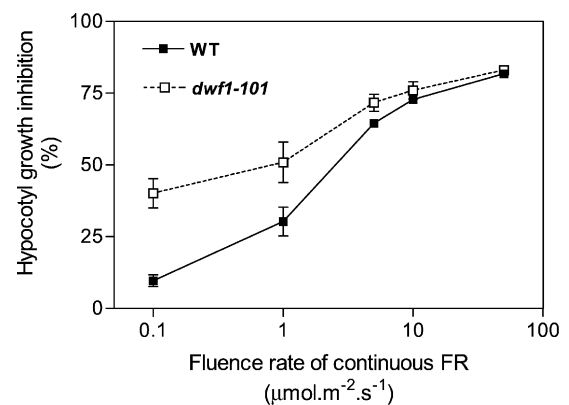


Figure 3. Reduced slope of the hypocotyl growth inhibition response to continuous FR in *dwf1-101*. Hypocotyl length in dark controls: WT = 11.4 ± 0.3 ; *dwf1-101* = 4.6 ± 0.3 . Data are means \pm SE of nine replicate boxes.

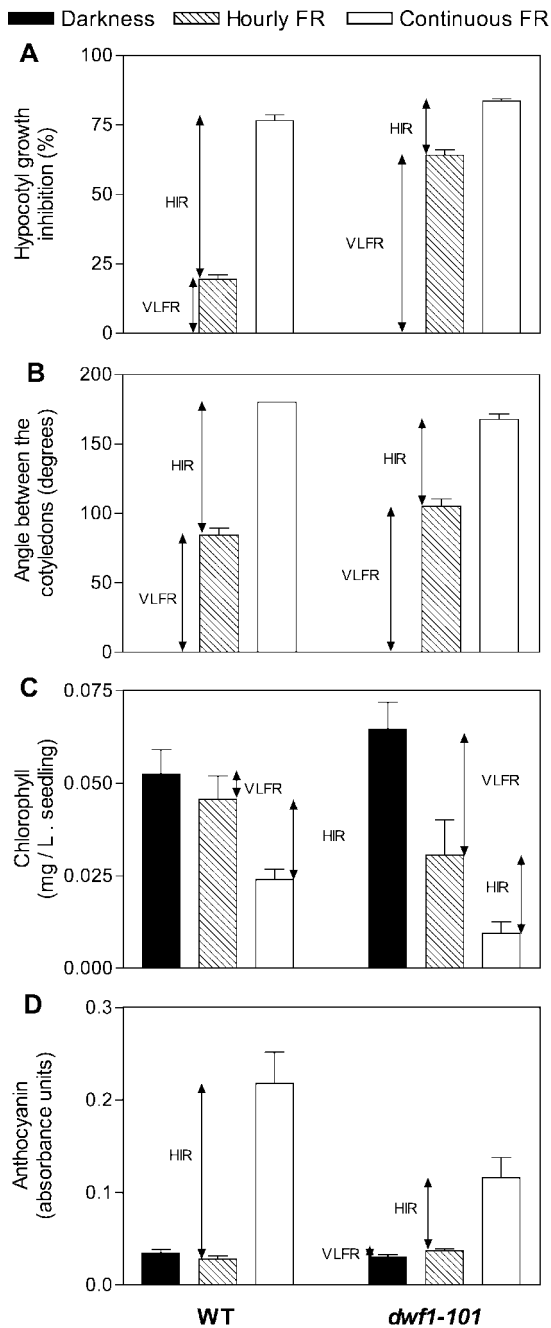


Figure 4. *dwf1-101* shows enhanced VLFR and reduced HIR of hypocotyl growth inhibition (A), cotyledon unfolding (B), blocking of greening (C), and anthocyanin synthesis (D). The seedlings were grown in darkness, hourly pulses of FR or continuous FR (at equal total fluence) before measurements or transfer to white light (chlorophyll experiments). Hypocotyl length in dark controls: WT = 8.4 ± 0.2 ; *dwf1-101* = 4.3 ± 0.2 . Data are means \pm SE of 12 (A and B), nine (C), or six (D) replicate boxes.

teroid levels, we analyzed the response to hourly FR in a previously isolated *dwf1/dim* allele (Takahashi et al., 1995; Klahre et al., 1998) and in *det2* (Li et al., 1996) both in the Columbia background. WT seedlings showed no significant cotyledon unfolding un-

der hourly FR and this is consistent with the deficient VLFR observed in the presence of Columbia alleles of the *VLF1* and *VLF2* loci (Yanovsky et al., 1997). *det2* also failed to unfold the cotyledons under hourly FR (Fig. 5A), but *dim* did show enhanced unfolding (angle between the cotyledons, degrees: WT = 0 ± 0 ; *dim* = 70 ± 10 ; $P < 0.01$). This suggests that for the VLFR phenotype, Columbia alleles of *VLF1* and *VLF2* loci are epistatic to a putative effect of *det2* but not to the effect of *dim*. In our hands *dim* had a stronger dark phenotype (shorter hypocotyls) than *det2* and this correlates with the relative impacts of these mutants on VLFR.

The lack of VLFR in *det2* Columbia offered the possibility to test whether the negative effects of *dwf1-101* on LFR and HIR can be observed in a genetic background where VLFR are not expressed. Cotyledon unfolding of seedlings exposed to hourly pulses of R or to continuous FR was reduced by the *det2* mutation ($P < 0.01$; Fig. 5A). A similar pattern was observed for hypocotyl growth inhibition (data not shown). This indicates that both LFR and HIR were negatively affected in *det2*.

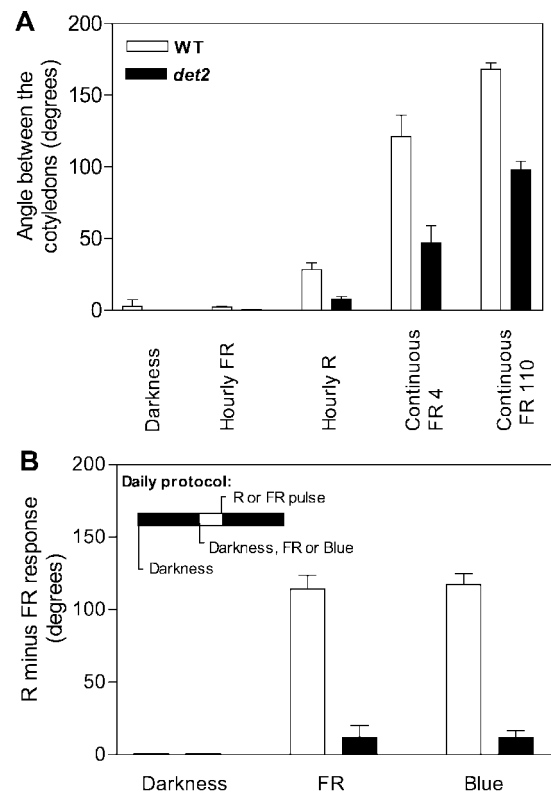


Figure 5. Reduced HIR and LFR in the brassinosteroid mutant *det2*. In A, the seedlings were grown in darkness or under hourly FR, hourly R, or continuous FR (4 or $100 \mu\text{mol m}^{-2} \text{s}^{-1}$). In B, the seedlings were daily exposed to a R versus a FR pulse given in factorial combination with 3 h of FR, 3 h of blue light, or darkness. The LFR (angle between the cotyledons for seedlings receiving a R pulse minus angle between the cotyledons for the seedlings receiving a FR pulse) is indicated for the 3 h of FR, 3 h of blue light, or no previous light conditions. Data are means \pm SE of 21 replicate boxes.

The LFR was further characterized by analyzing seedlings exposed daily to a R or a FR pulse (5 min) preceded by a blue light, FR or no photoperiod (3 h) because blue or FR pretreatments enhance the LFR mediated by *phyB* (Casal and Boccalandro, 1995). The difference in angle between cotyledons caused by terminal R versus FR pulses (i.e. the LFR) was negligible in the absence of the 3-h blue or FR exposure and was amplified by blue or FR photoperiods. The LFR response was reduced by the *det2* mutation ($P < 0.001$; Fig. 5B).

The Enhanced VLFR and the Reduced LFR of *dwf1-101* Depend on *phyA*

The *dwf1-101* mutant was crossed by the *phyA-201* null allele. The F_2 generation showed approximately a quarter of seedlings (23 of 97; $\chi^2 = 0.05$; $P > 0.8$) with fully folded cotyledons (angle below 10 degrees) under continuous FR. This is similar to the proportion observed in F_2 of crosses between WT and *phyA*. Approximately a quarter of the seedlings with closed cotyledons (5 of 23; $\chi^2 = 0.15$; $P > 0.7$) showed a hypocotyl shorter than expected for a *phyA* mutant but longer than expected for a seedling bearing *phyA*. Because the *dwf1-101* mutant shows intermediate hypocotyl length even in darkness, these seedlings were selected as homozygous *phyA dwf1-101* double mutants.

In the WT background, the VLFR of hypocotyl growth depends on the activity of *phyA* (Yanovsky et al., 1997; Fig. 6A). The *dwf1-101* mutant showed enhanced VLFR but this effect was abolished in the *phyA* background (note reduced hypocotyl growth inhibition in the *phyA dwf1-101* mutant for calculated Pfr/P at or below 10%; Fig. 6A). Noteworthy, even in the *phyA* background the *dwf1-101* mutation enhanced the response for Pfr/P = 20%. The photoreceptor mediating the latter residual effect remains to be elucidated. The slope of the LFR (Pfr/P higher than 30%) was reduced by the *dwf1-101* mutation in the WT background but not in the *phyA* background (Fig. 6A).

Cotyledon unfolding showed a similar pattern. Enhanced VLFR in the WT but not in the *phyA* background (angle between the cotyledons, degrees, for Pfr/P = 3%: WT = 5 ± 2 ; *dwf1-101* = 46 ± 5 ; $P < 0.0001$; *phyA* = 0 ± 0 ; *phyA dwf1-101* = 0 ± 0). Reduced LFR in the WT but not in the *phyA* background (Δ angle between the cotyledons between 33% and 61%, degrees: WT = 77 ± 7 ; *dwf1-101* = 39 ± 11 ; $P < 0.01$; *phyA* = 160 ± 6 ; *phyA dwf1-101* = 159 ± 8 ; $P > 0.9$).

The adult phenotype of the *phyA dwf1-101* and *phyB dwf1-101* double mutants was similar to the single *dwf1-101* mutant (Fig. 6B), indicating that neither *phyA* nor *phyB* are necessary for the *dwf1-101* effect at this stage.

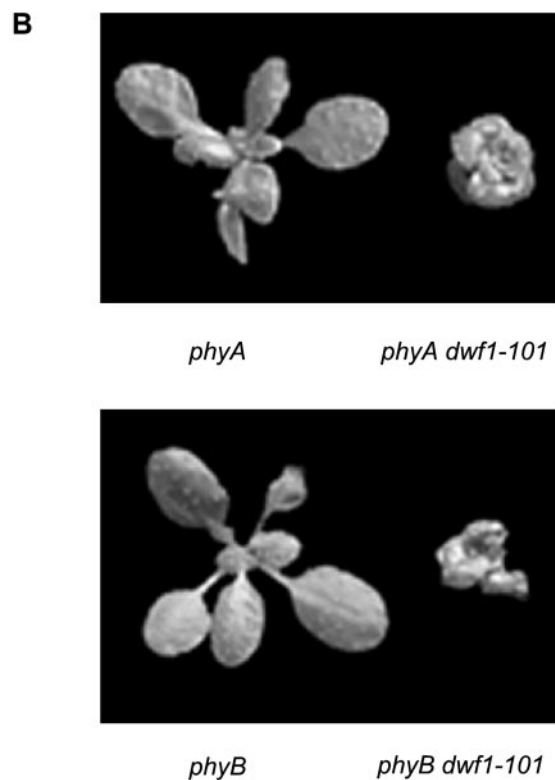
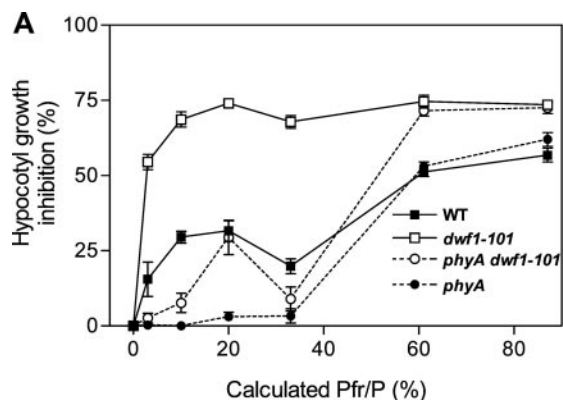


Figure 6. Seedling phenotype of the *phyA dwf1-101* double mutant (A) and adult phenotype of the *phyA dwf1-101* and *phyB dwf1-101* double mutants (B). In A, data are means \pm se of six replicates. Hypocotyl length in dark controls: WT = 9.7 ± 0.6 ; *dwf1-101* = 4.8 ± 0.1 ; *phyA* = 11.8 ± 0.5 ; *phyA dwf1-101* = 5.0 ± 0.3 .

Immunologically Detectable Levels of *phyA* Are Normal in *dwf1-101*

Because both the enhanced VLFR and the reduced LFR observed in *dwf1-101* depend on *phyA* (Fig. 6A), we investigated whether these effects were the result of alterations in *phyA* levels. A monoclonal antibody specific for *phyA* was used for this purpose. No significant differences were observed between WT and *dwf1-101* etiolated seedlings (Fig. 7).

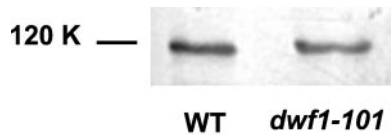


Figure 7. Normal levels of immunochemically detectable phyA in *dwf1-101*. The seedlings were grown in darkness for 4 d after the R pulse given for the induction of seed germination.

The *dwf1-101* Mutant Fails to Respond to Canopy Shadelight

Under laboratory conditions, the *dwf1-101* mutant showed enhanced VLFR and reduced LFR and HIR. To investigate the consequences of this altered photobiological behavior in terms of perception of natural light signals, the seedlings were grown under sunlight or canopy shadelight. The R/FR ratio beneath the canopy was 0.8 compared with 1.1 outside the canopy, and radiation within the visible range was reduced to a 14%. Despite the enhanced VLFR of the *dwf1-101* mutant, hypocotyl length was only slightly shorter than the WT under full sunlight conditions (Fig. 8). However, whereas the WT was taller under canopy shadelight than under full sunlight (a typical “shade-avoidance” response) the *dwf1-101* mutant failed to respond to the presence of a dense canopy (Fig. 8).

DISCUSSION

In systems like *Drosophila melanogaster*, re-isolation of mutants previously identified by means of a different protocol has provided useful insight into the complexity of regulatory interactions between pathways (e.g. Price et al., 1997). This is also beginning to be the case in *Arabidopsis* (e.g. Beaudoin et al., 2000). Here we have isolated *eve1*, a new allele of the *dim/dwf1* mutants that are deficient in brassinosteroid biosynthesis (Klahre et al., 1998; Choe et al., 1999).

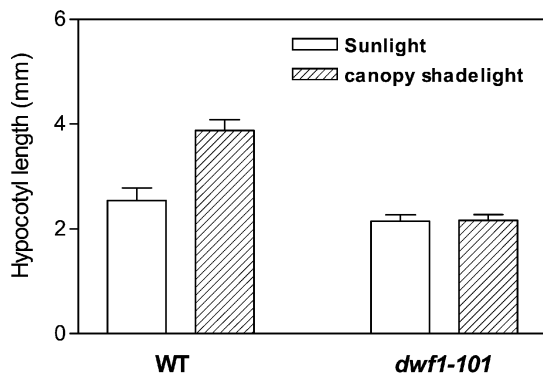


Figure 8. The *dwf1-101* mutant fails to respond to the presence of a shading canopy. The seedlings were grown in pots under sunlight or canopy shade light. The length of the hypocotyl (mm) in dark controls (grown near the other seedlings inside dark boxes) was: WT = 11.4 ± 0.7 ; *dwf1-101* = 8.1 ± 0.4 . Data are means \pm SE of 12 replicate plants.

dwf1-101 showed reduced hypocotyl length, increased cotyledon unfolding, increased anthocyanin levels, and blocking of greening under hourly FR. In darkness, hypocotyl length was reduced by the *dwf1-101* mutation but to a lesser extent than under pulsed FR. Cotyledon unfolding, anthocyanin synthesis and greening defects were not observed in dark controls. The *phyA dwf1-101* mutant failed to respond to pulsed FR indicating that in the WT, DWF1 is involved in the repression of VLFR mediated by phyA (Fig. 9). Therefore, we propose a role of DWF1 in down-regulation of VLFR.

The *bas1-D* mutant (Neff et al., 1999), which shows reduced brassinosteroid levels presumably because of enhanced steroid hormone inactivation by hydroxylation, exhibits hypersensitivity of hypocotyl growth inhibition to R, FR, and blue light. This effect of *bas1-D* is not reduced by a *phyB* mutation under R or by a *cry1* mutation under blue light, but (as observed here for *dwf1-101*) it is abolished by the *phyA* mutation under FR (Neff et al., 1999). Here we show that hypersensitivity is not restricted to hypocotyl growth inhibition but is specific to the VLFR mode of phyA signaling. The VLFR is predicted to operate under continuous R, FR, or blue light, because any of these light conditions exceeds the minimum requirements of VLFR. Thus, the behavior of *bas1-D* (Neff et al., 1999) and *dwf1-101* is consistent with a role of brassinosteroids in the repression of VLFR.

It is surprising that whereas VLFR were enhanced, LFR and HIR were partially repressed in *dwf1-101* and *det2* mutants. This uncovers a new role of brassinosteroids as positive regulators in the phytochrome signaling network. The latter conclusion is based on three complementary approaches to quantify LFR and HIR. After a photobiological approach, LFR and HIR were respectively calculated as the difference between either hourly R pulses or continuous FR and hourly pulses of FR. Hourly pulses of FR induce

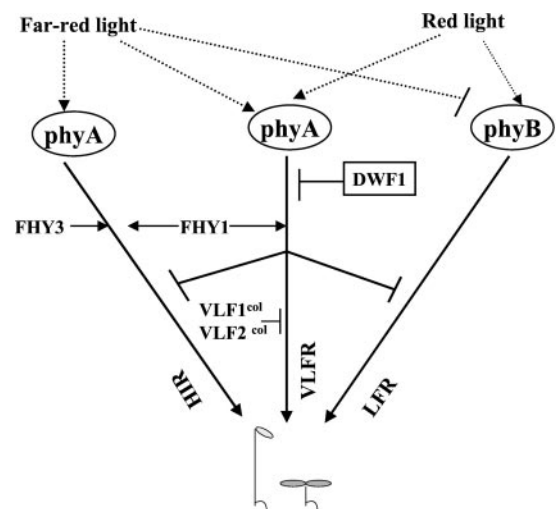


Figure 9. Model based on genetic and physiological data showing the proposed role of DWF1 in the phytochrome signaling network.

VLFR but provide neither enough Pfr to activate the LFR of phyB, nor the sustained activation required to elicit the HIR of phyA. After a genetic approach, LFR and HIR were analyzed without the interference of VLFR in the *det2* mutant, where VLFR were not observed because of the Columbia background (Yanovsky et al., 1997). Physiologically, HIR could be analyzed by using a response like anthocyanin synthesis where VLFR are negligible. The three approaches consistently showed reduced LFR and/or HIR in brassinosteroid mutants.

We had previously observed that *phyA* and *fhy1* mutants have enhanced phyB-mediated responses to R (Mazzella et al., 1997; Cerdán et al., 1999) whereas Columbia alleles of the *VLFR* loci reduce VLFR but do not enhance phyB-mediated responses (Yanovsky et al., 1997). These observations have been interpreted as a negative regulation of phyB signaling exerted by elements of the phyA-FHY1 VLFR pathway upstream the point of action of *VLFR1* and *VLFR2* (Fig. 9). The positive effect of DWF1 and DET2 on phyB signaling required phyA signaling in the VLFR, as indicated by the similar LFR in *phyA* and *phyA dwf1-101* mutants (Fig. 6A). The positive effect of DET2 was not abolished even in the Columbia background (Fig. 5). Thus, we propose that brassinosteroids down-regulate early steps of the VLFR signaling pathway upstream the action of *VLFR* loci and this results in amplification of phyB-mediated signaling (Fig. 9).

Although brassinosteroid mutants also have reduced HIR, the dependence of this regulation on VLFR signaling cannot be tested by using a *phyA* mutant to eliminate VLFR because these mutants also lack HIR. However, we have observed that transgenic plants that overexpress phyA have enhanced VLFR and reduced HIR (J.J. Casal, S.J. Davis, M.J. Yanovsky, R.C. Clough, E.T. Jordan-Beebe, and R.D. Vierstra, unpublished data). Thus, we have tentatively included a negative link between VLFR and HIR (Fig. 9) to account for the reduced HIR in *dwf1-101* and *det2*.

Present results indicate a role of brassinosteroids in fine tuning of phytochrome-mediated responses. Brassinosteroids would shift the sensitivity from the range of weak light signals versus darkness (experienced by seeds during soil tillage or etiolated seedlings close to the surface of the soil) to the range of modifications in R/FR ratio and irradiance caused by neighbor plants. The significance of this regulation is highlighted by the impaired responses to canopy shadelight in *dwf1-101* (Fig. 8). Thus, changes in brassinosteroid levels would help to adjust plant sensitivity to different light signals according to the developmental and environmental context. Light down-regulates a small G protein, which in turn acts positively on a variant P450 that catalyzes C-2 hydroxylation in brassinosteroid biosynthesis (Kang et al., 2001). This mutual influence, where brassinosteroids regulate light responses and light regulates

brassinosteroid levels, could play a key role in the dialog between environmental and endogenous cues controlling plant development.

MATERIALS AND METHODS

Plant Material

Mutagenized seed of Arabidopsis of the ecotype Landsberg *erecta* was purchased from Lehle Seeds (Round Rock, TX). For the mutant screening the seeds were incubated in boxes (175 × 225 mm² and 45-mm height) containing 0.8% (w/v) agar for 3 d at 7°C before transfer to the specific protocol conditions. The WT was Landsberg *erecta*. The *dim/dwf1-2* mutant (Takahashi et al., 1995; Klahre et al., 1998; Choe et al., 1999) was used for complementation analysis and in unreported physiological experiments (compared with the WT Columbia). The *det2* mutant (Li et al., 1996) was compared with the WT Columbia in physiological experiments. Seed samples of *dim* and *det2* were provided by the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The *phyA-201* (Nagatani et al., 1993) and *phyB-5* (Reed et al., 1993) were used to obtain double mutants.

For laboratory experiments with *eve1 (dwf1-101)*, *det2*, or *dim*, seeds of Arabidopsis were sown in clear plastic boxes (40 × 33 mm² × 15-mm height) containing 3 mL of 0.8% (w/v) agar. The number of seeds per box was 15, 50, or 200 in morphological, chlorophyll, and anthocyanin experiments, respectively. The seeds were incubated in darkness at 7°C for 3 d, given a R pulse to promote seed germination, and incubated in darkness (25°C) for 24 h before light treatments. For greenhouse experiments, the seedlings were sown in pots (35-mm diameter, 7.5-mm height) containing soil. The seeds were chilled and induced to germinate as described for laboratory experiments.

Hypocotyl Growth and Cotyledon Unfolding

The seedlings were exposed either to hourly pulses of R, FR, or R plus FR mixtures (3 min, 15–40 μmol m⁻² s⁻¹); these fluence rates saturate the response to the pulses that provided a series of calculated Pfr/P (for details of light sources, spectral distribution and Pfr/P calculations, see Casal et al., 1991; Yanovsky et al., 2000), or to continuous FR (calculated Pfr/P = 10%, fluence rates between 0.1 and 100 μmol m⁻² s⁻¹), whereas control seedlings remained in darkness. In some experiments, hourly and continuous FR were compared at equal total fluence (36 mmol m⁻² h⁻¹). To amplify the LFR mediated by phyB, in some experiments with the *det2* mutant the seedlings were daily exposed to 3 h FR or blue light (40 μmol m⁻² s⁻¹) provided by fluorescent lamps in combination with a 2-mm-thick blue acrylic filter. Hypocotyl length was measured to the nearest 0.5 mm with a ruler in the 10 longest seedlings (this eliminates defective seedlings). The angle between the cotyledons was measured in the same seedlings with a protractor. Seedling data were averaged per box (one replicate) and used for statistics.

In greenhouse experiments, the pots were placed under sunlight (photoperiod 14 h), under a dense canopy of tomato plants or in complete darkness (inside a box wrapped in aluminum foil). The R/FR ratio was measured with a Skye SKR 110 sensor (Skye Instruments Ltd, Llandrindod Wells, Powys, UK). Four days after transfer to the greenhouse, the seedlings were removed from the soil and hypocotyl length was measured to the nearest 0.5 mm with a ruler.

Chlorophyll and Anthocyanin Levels

For blocking of greening experiments, 24 h after the R pulse to induce germination, the seedlings were transferred to hourly pulses (3 min, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$) or continuous ($2 \mu\text{mol m}^{-2} \text{s}^{-1}$) long wavelength FR (Pfr/P = 3%) provided by an incandescent lamp in combination with a water filter and an RG9 filter (Schott, Mainz, Germany), or remained in darkness. Three days later, the seedlings were transferred to continuous fluorescent white light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 2 d (note that in previous experiments we used 1 d and this results in different chlorophyll background levels; Yanovsky et al., 2000). The seedlings were harvested in *N,N'*-dimethylformamide and incubated in darkness at -20°C for at least 3 d. Absorbance was measured at 647 and 664 nm, and chlorophyll levels were calculated according to Moran (1982).

For anthocyanin experiments, the seedlings were exposed for 3 d to hourly pulses (3 min) or continuous FR (calculated Pfr/P = 10%; $36 \text{ mmol m}^{-2} \text{h}^{-1}$) and subsequently extracted with 1 mL of 1% (w/v) HCl methanol. Measurements of A_{530} were corrected for chlorophyll absorption (657 nm) according to Mancinelli et al. (1991).

Immunochemical Detection of phyA and phyB

Extracts were prepared from samples harvested on ice according to Martínez-García et al. (1999). The extracts were subjected to SDS-PAGE in 1.5-mm thick, 4.5%/7.5% stacking/resolving gel (Mini Protean II, Bio-Rad, Richmond, CA). Proteins were electroblotted to nitrocellulose (0.45- μm pore size, Sigma, St Louis) following manufacturer's indications. The remaining protein-binding capacity was blocked with 5% (w/v) skim milk, 50 mM Tris-Cl, and 200 mM NaCl, pH 7.4 for 30 min at 37°C . The anti phyA monoclonal antibody 073D raised in mouse against purified phytochrome from etiolated oats was kindly provided by Dr. Peter H. Quail (University of California, Berkeley, and U.S. Department of Agriculture Plant Gene Expression Center, Albany, CA). The blots were incubated overnight at 4°C with this primary antibody at a dilution of 1:1,000. After washing, the membrane was incubated with 1:500 affinity isolated alkaline-phosphatase-conjugated antibody to mouse IgG developed in goat (Sigma). The bands were visualized by incubating the blots in 0.1 M Tris (pH 9.5), 100 mM NaCl, and 5 mM MgCl_2 containing 0.165 mg ml^{-1} 5-bromo-4-chloro-3-indoyle phosphate, *p*-toluidine salt, and 0.33 mg ml^{-1} nitroblue tetrazolium (Sigma).

Mapping

A mapping population was generated by crossing the *eve1* mutant in Landsberg *erecta* with the Columbia ecotype. DNA was isolated from 85 plants showing compact rosettes and reduced stature following the protocol described by Rogers and Bendich (1988). Markers for simple sequence length polymorphisms (Arabidopsis database Stanford University, Palo Alto, CA; <http://www.Arabidopsis.org>) were used to map the position of *eve1*.

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