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Phytochrome A Mediates the Promotion of Seed Germination by Very Low Fluences of Light and Canopy Shade Light in Arabidopsis[1]

Javier F. Botto, Rodolfo A. Sánchez, Garry C. Whitelam, and Jorge J. Casal*

Ifeva, Departamento de Ecología, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, 1417-Buenos Aires, Argentina (J.F.B., R.A.S., J.J.C.); and Department of Botany, University of Leicester, Leicester LE1 7RH, United Kingdom (G.C.W.)

Seeds of the wild type (WT) and of the *phyA* and *phyB* mutants of *Arabidopsis thaliana* were exposed to single red light (R)/far-red light (FR) pulses predicted to establish a series of calculated phytochrome photoequilibria (Pfr/P). WT and *phyB* seeds showed biphasic responses to Pfr/P. The first phase, i.e. the very-low-fluence response (VLFR), occurred below Pfr/P = 10^{-1}%. The second phase, i.e. the low-fluence response, occurred above Pfr/P = 3%. The VLFR was similarly induced by either a FR pulse saturating photoconversion or a subsaturating R pulse predicted to establish the same Pfr/P. The VLFR was absent in *phyA* seeds, which showed a strong low-fluence response. In the field, even brief exposures to the very low fluences of canopy shade light (R/FR ratio < 0.05) promoted germination above dark controls in WT and *phyB* seeds but not in the *phyA* mutant. Seeds of the *phyA* mutant germinated normally under canopies providing higher R/FR ratios or under deep canopy shade light supplemented with R from light-emitting diodes. We propose that phytochrome A mediates VLFR of *A. thaliana* seeds.

Seed germination in the field depends on many environmental circumstances, including the position of the seeds within the soil profile, soil disturbances, the extent of canopy cover, etc. (Egley, 1995). In a large number of species, these responses are at least partially mediated by phytochrome perception of the light environment prevailing under such conditions (Vázquez-Yañez and Smith, 1982; Scopel et al., 1991; Deregibus et al., 1994; Insausti et al., 1995). Phytochrome exists in two photo-interconvertible forms, Pr and Pfr, that absorb maximally in R and FR, respectively (Kendrick and Kronenberg, 1994). Photoconversion of Pr to Pfr (normally considered as the active form of phytochrome) promotes germination, and this promotion is often canceled by subsequent exposure to FR that converts phytochrome back to Pr (normally considered the inactive form). However, in highly sensitive seeds FR causes only partial reversal of the R effect, and FR alone significantly promotes germination above dark controls (VanDerWoude and Toole, 1980; Cone et al., 1985). One of the possible interpretations of the latter phenomenon is that these seeds require only small amounts of Pfr to germinate and are promoted by the Pfr levels remaining after FR. This idea looks convincing but there is one important caveat—namely, that the involvement of phytochrome in the promotion of seed germination by FR cannot be tested using the classical physiological test of R/FR reversibility.

Sensitization can be achieved by laboratory treatments, including chilling (VanDerWoude and Toole, 1980; VanDerWoude 1985), heating (Taylorson and Dinola, 1989), temperature shifts (Cone et al., 1985), GA_3 (Rethy et al., 1987), anesthetics (VanDerWoude, 1985), or incubation in water vapor phase (A.L. Scopel, unpublished data). Sensitization also occurs under field conditions. Buried seeds of the annual weed *Datura ferox* exhibit a 10,000-fold increase in light sensitivity (compared to seeds stored dry) and can be induced to germinate by the very brief exposures to sunlight that take place during soil cultivations (Scopel et al., 1991). Seeds in the soil can display wide seasonal changes in sensitivity to light, as Derkx and Karssen (1993) found in buried seeds of *Sisymbrium officinale*.

Under certain conditions, germination responses to the fluence of a R pulse are biphasic (Cone et al., 1985; VanDerWoude, 1985; Rethy et al., 1987; Scopel et al., 1991; Derkx and Karssen, 1993). The first phase, called VLFR, shows a plateau that coincides with germination levels induced by a saturating FR pulse. The second phase, called LFR, is the classical R/FR reversible response.

There are different hypotheses (reviewed by Cone et al., 1985) to explain the biphasic curves (of germination and other processes). VanDerWoude (1985), for instance, proposed that VLFR are induced by Pr-Pfr dimers and LFR by Pfr-Pfr dimers. Hecht and Mohr (1990) reported evidence against this view and proposed the occurrence of multiple primary actions of phytochrome.

Plants possess multiple, discrete molecular species of phytochrome whose apoproteins are coded by a small family of divergent genes (Quail, 1994). Five phytochome genes, *PHYA* through *PHYE*, are present in *Arabidopsis*

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* Corresponding author; e-mail jjcasal@criba.edu.ar; fax 541–521–1384.

Abbreviations: FR, far-red light; HIR, high-irradiance response; LFR, low-fluence response; Pfr/P, proportion of phytochrome in its far-red-light-absorbing form; phyA, phytochrome A; phyB, phytochrome B; R, red light; VLFR, very-low-fluence response; WT, wild type.

thaliana (Sharrock and Quail, 1989; Clack et al., 1994). Different phytochrome action modes (VLFR, LFR, and HIR) could be mediated by different phytochromes. Arabidopsis mutants specifically lacking phyA fail to exhibit the HIR, causing severe hypocotyl growth inhibition under continuous FR (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), whereas overexpressed phyA enhances stem-growth HIR (McCormac et al., 1993). Overexpression of *Avena* phyA in etiolated transgenic tobacco seedlings also increases VLFR but has very little effect on LFR (Casal et al., 1994). Arabidopsis (Koornneef et al., 1980; Nagatani et al., 1991; Somers et al., 1991; Reed et al., 1993) and cucumber (López-Juez et al., 1992) mutants deficient in phyB do not respond to end-of-day FR, which may be classified as a LFR, but show normal responses under continuous FR (Reed et al., 1994; Shinomura et al., 1994). Germination of *phyB* mutant seeds of Arabidopsis shows normal VLFR and reduced LFR (Botto et al., 1995).

PhyA is involved in germination responses under continuous FR (Reed et al., 1994) or pulsed FR (Johnson et al., 1994), but *phyA* mutant seeds germinate normally in response to a R pulse, suggesting that phyA could mediate VLFR. In the work described here, we used seeds of the WT, *phyA-1*, and *phyB-1* mutants of *A. thaliana* to determine if phyA-mediated germination responses (a) can be ascribed to a particular phytochrome action mode and (b) participate in the control of germination under natural radiation.

MATERIALS AND METHODS

Laboratory Experiments

Seeds of WT (ecotype Landsberg erecta), *phyA-1* mutant (Whitelam et al., 1993), and *phyB-1* mutant (Koornneef et al., 1980) of *Arabidopsis thaliana* (L.) Heynh. were harvested from mature fruits of plants grown under continuous fluorescent white light (PPFD = 100 μmol m^{-2} s^{-1}) at 25°C. Before the experiments, seeds were stored in darkness at 25°C.

Samples of 25 seeds were placed in clear plastic boxes (42 × 35 mm^2 × 20 mm) on two layers of filter paper (0.1 mm thickness) moistened with 0.8 mL of distilled water. The boxes, wrapped in black plastic sheets, were incubated for 3 d at 7°C followed by 8 h at 35°C and equilibrated (0.5 h) at 25°C, immediately prior to the irradiation (Cone and Kendrick, 1985; Botto et al., 1995). The handling of the seeds was performed in absolute darkness. The seeds were exposed to a single saturating or subsaturating R or FR pulse. Intermediate calculated Pfr/P was provided by different R + FR mixtures. At equal calculated Pfr/P, similar percentages of germination were obtained by using R + FR mixtures or interference filters (Botto et al., 1995). Details of construction of the light sources were as described earlier (Casal et al., 1991; Botto et al., 1995). Light-emitting diodes with maximum emission at 662 nm (band width at 0.5 maximum emission = 24 nm) in combination with neutral filters were used for subsaturating R pulses (18 μmol m^{-2}). Scans of the light sources were obtained with a Li-Cor (Lincoln, NE) 1800 calibrated spectroradiometer. Pfr/P values were calculated, after correcting the scans of the light sources by seed-coat transmittance, as described earlier (Botto et al., 1995). After light treatments, the seeds were incubated in darkness for 4 d at 20°C before germination was recorded. Radicle protrusion was taken as criterion for germination. The percentage of germinating seeds was transformed using probits (Finney, 1952), because within each response phase, the relationship between the logarithm of Pfr and the probit of germination is linear (Cone and Kendrick, 1985, 1986).

Field Experiments

Samples of 25 seeds (obtained as described for laboratory experiments) were sown in cylindrical plastic pots (40 mm height, 15 mm diameter) containing a soil-sand mixture. Groups of 21 pots were placed in each clear plastic box (140 × 75 mm^2 × 70 mm height) containing 50 mL of distilled water. The tops of the boxes were covered with a transparent plastic sheet to eliminate predators. The boxes were placed under canopies of *Arundo donax* or *Medicago sativa*. The R/FR ratio was measured at the bottom of the canopies with a Skye sensor (SKR 100, remote probe SKR 110, Llandrindod Weels, Powys, UK). Air temperature inside and outside the boxes was recorded with thermistor probes (107b, Campbell Scientific, Logan, UT) connected to a micrologger (21X, Campbell Scientific). Dark control boxes were wrapped in black plastic sheets and placed close to the boxes exposed to canopy light. In some experiments, supplementary R (0.06 μmol m^{-2} s^{-1}; maximum emission at 660 nm; band width at 0.5 maximum emission = 40 nm) was provided by light-emitting diodes (seven per box) placed inside the boxes, 5 cm above the seeds. Unless stated otherwise, germination was recorded after 4 d of treatment.

RESULTS AND DISCUSSION

The VLFR Is Absent in phyA Seeds

Seeds of WT, *phyA*, and *phyB* Arabidopsis stored dry at 25°C for 1 to 6 weeks and subsequently pretreated for 3 d at 7°C and 8 h at 35°C showed reduced germination in darkness (Fig. 1). In seeds of the WT and of the *phyB* mutant, germination was promoted by a R light pulse (Pfr/P = 85%) and, to a lesser degree, by a FR light pulse (Pfr/P = 2.9%). In contrast, germination of *phyA* mutant seeds responded to R but was not promoted by a FR pulse.

In another set of experiments, seeds were stored dry for 22 weeks, pretreated as indicated above, and exposed to a single light pulse (saturating R, FR, or R + FR mixtures, and subsaturating FR) predicted to establish a series of Pfr/P. Germination responses of WT and *phyB* seeds plotted against Pfr/P showed two phases separated by a plateau (Fig. 2A) (Cone et al., 1985; Botto et al., 1995). The VLFR occurred below Pfr/P = 10^{-1}% (Fig. 2B). The LFR occurred between Pfr/P = 3 and Pfr/P = 85%. In *phyA* seeds the VLFR was absent and the LFR was even steeper than in the WT (Fig. 2).

Overexpression of phyA in etiolated transgenic seedlings of tobacco increases cotyledon unfolding in the VLFR re-

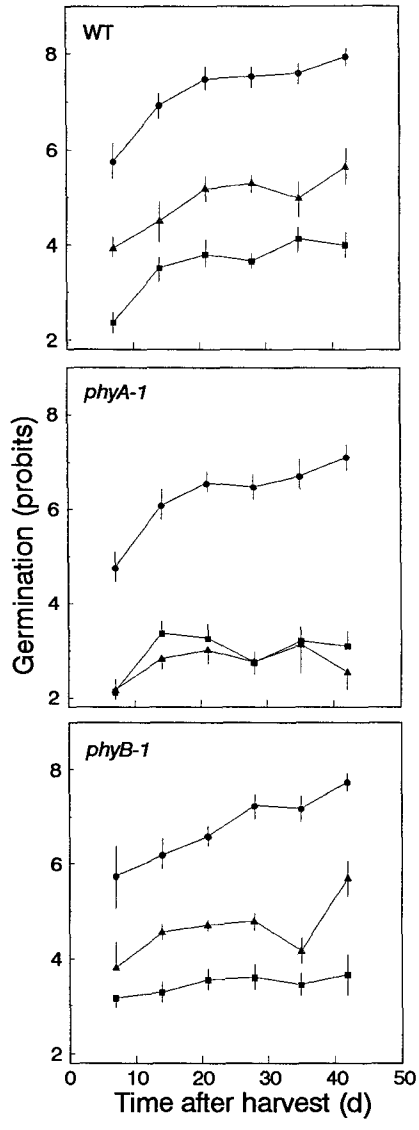

Figure 1. Germination of WT, *phyA-1*, and *phyB-1* seeds of *A. thaliana* as affected by a R pulse (●), a FR pulse (▲), or darkness (■) at different times after harvest. Seeds were stored dry at 25°C, incubated for 3 d at 7°C, followed by 8 h at 35°C, and exposed to the light pulse. Germination was recorded after 4 d. Data are means of 12 replicates (involving 4 different batches of seeds) ± SE.

gion but has little effect in the LFR region (Casal et al., 1994). In etiolated seedlings of Arabidopsis, hourly FR pulses (calculated Pfr/P = 3%) inhibit hypocotyl growth in the WT but not in the *phyA* mutant, whereas hourly R pulses (calculated Pfr/P = 87%) cause larger inhibitory effects in the mutant than in the WT (Casal, 1995). These observations are consistent with the idea that VLFR are the inductive responses (i.e. responses to light pulses, as opposed to those responses only occurring under continuous light) mediated by phyA. It appears that phyA, at most, makes a poor contribution to the promotion of cotyledon unfolding and seed germination in the LFR region. This selective effect of phyA suggests that VLFR and LFR could be the manifestation of two primary sites of phytochrome action with differential affinity by phyA (Casal et al., 1994).

phyA Mediates Responses to R and FR

In etiolated seedlings, de-etiolation is largely mediated by phyB under continuous irradiation with R and by phyA under continuous FR, indicating reciprocal responsivities of these photoreceptors (Quail et al., 1995). The reasons for this selective action of phyA and phyB have not been unequivocally established. To investigate whether phyA is selectively activated by R or FR when provided as brief pulses, Arabidopsis seeds were exposed to a FR pulse saturating photoconversion or a subsaturating R pulse, both predicted to establish the same calculated Pfr/P (2.9%) corresponding to the VLFR region. In WT and *phyB* seeds, germination was similarly promoted by either a saturating FR pulse or a subsaturating R pulse. Neither light treatment was effective in *phyA* seeds (Fig. 3). Thus,

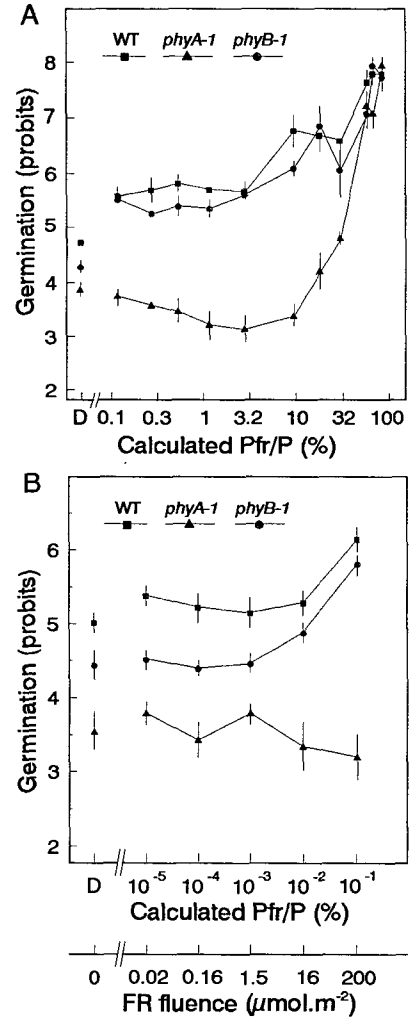

Figure 2. A, Germination of WT, *phyA-1*, and *phyB-1* seeds of *A. thaliana* plotted against the calculated Pfr/P provided by a light pulse. B, Details of VLFR. Data are means of nine (A) or eight (B) replicates ± SE. Protocol as in Figure 1.

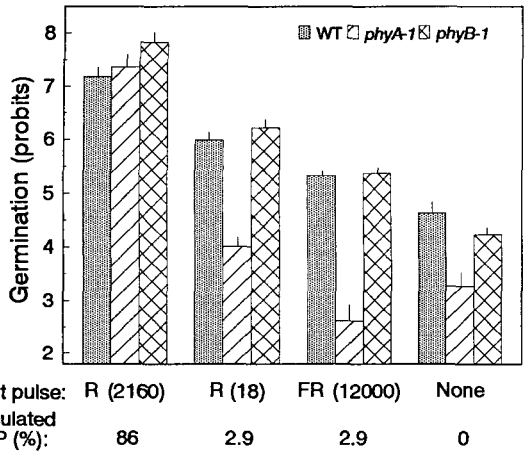

Figure 3. Germination of WT, *phyA-1*, and *phyB-1* seeds of *A. thaliana* as affected by either a FR or a R pulse predicted to establish the same Pfr/P within the VLFR region. The total fluence (μmol m^{-2}) is indicated in parentheses. Data are means of 8 to 12 replicates ± SE. Protocol as in Figure 1.

although for de-etiolation responses the role of phyA is obvious under continuous FR and not under continuous R, the VLFR of seed germination is similarly mediated by phyA after a R or a FR pulse.

In the *phyA* mutant FR pulses slightly, but consistently, reduced germination below the levels of dark control seeds (Figs. 2 and 3). A subsaturating R pulse caused no effect compared to dark controls (Fig. 3). These observations are consistent with the idea that FR reduces the percentage of germination in *phyA* seeds by phototransforming some stable pool from Pfr (remaining in the seed) to Pr.

Reduced Germination of phyA Seeds under Dense Canopies

Seeds of the WT and of the *phyB* mutant showed high germination rates when exposed to the deep shade light (R/FR = 0.05) of a dense canopy (Fig. 4). Seeds incubated in darkness beneath the same canopy germinated poorly. No differences in temperature (maximum average = 25.9°C, minimum average = 18.2°C) were recorded between the boxes incubated in darkness or exposed to light (both beneath the canopy). Thus, the promotion of seed germination can be ascribed to the light environment. Deep canopy shade light had a small effect in the *phyA* mutant (Fig. 4). In several species the low R/FR ratios of canopy shade light inhibit germination compared to the high R/FR ratios found in open places (e.g. Deregibus et al., 1994; Insausti et al., 1995), and this is currently thought to be a widespread response involved in gap sensing (Fenner, 1995). In WT Arabidopsis (at least under the present conditions), the seeds would be able to perceive light changes associated with their position on or below the surface of the soil but not the degree of canopy cover. It must be emphasized that the seeds used here were harvested from plants grown in cabinets, under artificial lighting, and stored dry at 25°C. It is not known whether "field" seeds actually germinate under dense canopies. Thus, although the ability of phyA to perceive deep canopy shade light is unequivocally established by the present experiments, it would be premature to attach any fitness significance to this finding.

Slight increases in the R/FR ratio (e.g. from <0.05 to 0.15) were enough to induce almost normal germination rates in *phyA* seeds (Fig. 5). Supplementary R, provided by light-emitting diodes, restored high germination rates in *phyA* seeds beneath very dense canopies, i.e. seeds that otherwise would be exposed to inhibitory R/FR ratios (Fig. 5, open symbols).

Pulses of Canopy Shade Light Promote Germination

Under laboratory conditions, phyA mediates VLFR (Fig. 2). In field experiments, phyA mediates the promotion of seed germination by deep canopy shade light compared to darkness (Figs. 4 and 5). In both situations, phyA is involved when the treatments are predicted to establish very low proportions of Pfr, although light pulses were used in

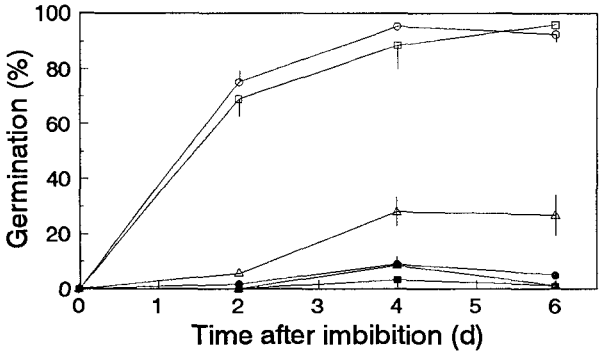

Figure 4. Germination of WT (□), *phyA-1* (△), and *phyB-1* (○) seeds of *A. thaliana* placed beneath a dense cane canopy (R/FR ratio = 0.05). Dark control seeds (■, WT; ▲, *phyA-1*; ●, *phyB-1*) were also incubated beneath the canopy. Data are means of seven replicates ± SE.

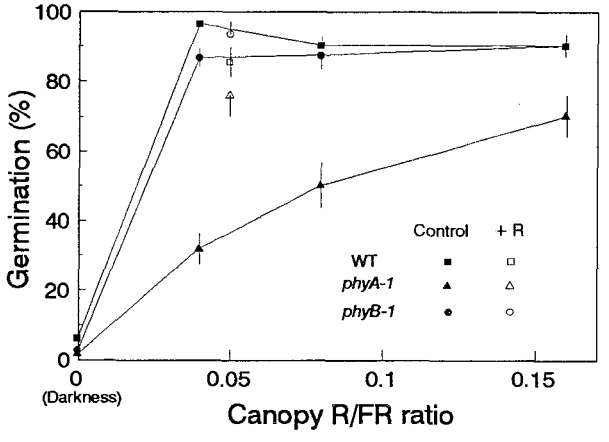

Figure 5. Germination of WT, *phyA-1*, and *phyB-1* seeds of *A. thaliana* placed beneath cane canopies providing different R/FR ratios. Open symbols, Seeds placed under canopy light (of the R/FR indicated in abscissa) supplemented with R provided by light-emitting diodes. Filled in symbols, Controls. Data are means of seven replicates ± SE.

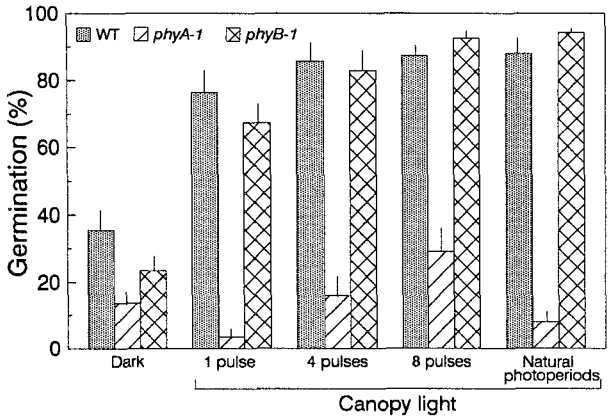

Figure 6. Germination of WT, *phyA-1*, and *phyB-1* seeds of *A. thaliana* placed beneath a dense alfalfa canopy as affected by brief (0.5 h) exposures to shade light (R/FR ratio = 0.04). 1 pulse, Exposure occurred after 3 h of imbibition. 4 pulses, One pulse per day (the first after 3 h of imbibition). 8 pulses, Two pulses per day. The rest of the time the boxes with the pots containing seeds remained beneath the canopy, wrapped in black plastic sheets. Data are means of seven replicates ± SE.

the laboratory and normal photoperiods were used in the field. To investigate the possible relationship between these phenomena, the seeds were exposed to pulses of canopy shade light. A single pulse of canopy shade light strongly promoted germination in seeds having phyA (Fig. 6). This observation is consistent with the involvement of VLFR in the promotion of seed germination by deep canopy shade light.

CONCLUSIONS

Photomorphogenesis research faces heterogeneity at different levels. There are different light signals, different phytochromes, different phytochrome action modes, and different responses. The results presented here establish links among some of these levels. First, the promotion of seed germination mediated by light absorbed by phyA operates via the VLFR mode. Second, Arabidopsis seeds harvested and stored under laboratory conditions germinate under very dense canopies via phyA perception of shade light.

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