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PHOTOINDUCTION OF SEED GERMINATION IN *ARABIDOPSIS THALIANA* IS MODULATED BY PHOTOTROPINS

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Light exposure is an important environmental factor which breaks seed dormancy in many plant species. Phytochromes have been identified as playing a crucial role in perception of the light signal that releases seed germination in *Arabidopsis*. Phototropins (Phot1, Phot2) are blue/UV-photoreceptors in plants which mediate phototropic responses, chloroplast relocation, hypocotyl growth inhibition and stomata opening. We studied germination under different light conditions in *Arabidopsis* Phot1-null and Phot2-null mutants and in a double *phot1phot2* mutant. Germination of single *phot1* and *phot2* mutants in darkness was much lower than in wild-type (WT) seeds, whereas double *phot1phot2* mutant lacking both functional phototropins germinated at frequency comparable to WT seeds, irrespective of light and temperature conditions. Light treatment of imbibed seeds was essential for effective germination of *phot1*, irrespective of low-temperature conditioning. In contrast, cold stratification promoted dark germination of *phot2* seeds after imbibition in dim light. Low germination frequency of *phot1* seeds under low light intensity suggests that the presence of functional Phot1 might be crucial for effective germination at these conditions. The lower germination frequency of *phot2* seeds under continuous light suggests that Phot2 might be responsible for stimulating germination of seeds exposed to direct daylight. Thus, the phototropin system may cooperate with phytochromes regulating the germination competence of seeds under different environmental conditions.

Key words: Photomorphogenesis, seed germination, phototropins, light signalling, *Arabidopsis thaliana*.

INTRODUCTION

In many plant species the transition of seeds from dormancy to germination is controlled by environmental factors. In *Arabidopsis*, most important are light exposure and temperature decrease (Finch-Savage et al., 2006; Holdsworth et al., 2008). Phytochromes have been shown to play a crucial role in the control of germination in *Arabidopsis* (for reviews see: Casal and Sanchez, 1998; Bae and Choi 2008). In particular, phytochrome B has been identified as a photoreceptor involved in promotion of germination of dark-imbibed seeds by a pulse of red light, through a classical red-far red reversible low-fluence response (LFR) mode (Casal and Sanchez, 1998). Action spectroscopy experiments with mutants that lack functional phytochrome A (*phyA*) and phytochrome B (*phyB*) demonstrated that phytochrome A is a photoreceptor involved in promotion of germination via the very low fluence response (VLFR) (Shinomura et al., 1996). More recently, phytochrome E was shown to be indispensable for induction of seed germination under continuous far red (FR) light (Hennig et al., 2002), and the phytochrome-interacting basic helix-loop-helix protein PIL5 has been identified as a key negative regulator of seed germination (Oh et al., 2004).

Phototropins are blue/UV-A photoreceptors in plants, characterized recently (Briggs and Christie, 2002; Christie, 2007). Two members of the phototropin family present in *Arabidopsis*, Phot1 and Phot2, showing close sequence similarity, have been demonstrated to initiate the phototropic responses of hypocotyls and stems (Liscum and Briggs, 1995; Huala et al., 1997; Sakai et al., 2001) and blue-light-induced chloroplast relocation in mesophyll cells (Jarillo et al., 2001; Kagawa and Wada, 2000). Phototropins play a role in such diverse plant processes as stomata opening (Kinoshita et al., 2001), leaf expansion (Sakamoto and Briggs, 2002),

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hypocotyl growth inhibition (Folta and Spalding, 2001) and light-induced mRNA destabilization in greening cotyledons (Folta and Kaufman, 2003).

In this work we quantitatively analyzed germination under environmentally realistic light and temperature conditions in *phot1* and *phot2* mutants and in a double *phot1phot2* mutant lacking functional phototropins. Our results suggest that phototropins 1 and 2 are involved in regulation of the transition from dormancy to germination in *Arabidopsis* seeds.

MATERIALS AND METHODS

PLANT MATERIAL AND SEED CULTURE

The *Arabidopsis thaliana* wild-type line of the Columbia ecotype (Col-0) was obtained from the *Arabidopsis* Biological Resource Center, Ohio State University, USA. The mutant lines used in this work were *phot1* (Liscum and Briggs, 1995), *phot2* (Jarillo et al., 2001) and a double *phot1phot2* mutant. The seed batches used in all experiments were obtained from plants grown under standardized conditions in a conditioned growth chamber at constant 22±2°C and 65% humidity under a 12 h photoperiod (Sylvania Luxline Plus daylight fluorescent lamps, fluence 80–120 μmol $m^{-2}s^{-1}$). At least two independent seed batches were used for germination tests.

LIGHT TREATMENT AND GERMINATION ASSAY

White light (WL, 60 μmol $m^{-2}s^{-1}$) was delivered by Osram fluorescent tubes (36 W/20). Light fluence was measured with an SKP 215 PAR quantum sensor (Skye Instruments Ltd., UK).

For experiments including imbibition under light, wild-type and mutant seeds were sterilized for 20 min in 3% hypochlorite with 0.1% Triton X-100 added (Serva, Germany), washed thoroughly with sterile deionized water and sown in Petri dishes (typically 50–150 seeds of each tested plant line per plate) containing Murashige and Skoog medium (Sigma-Aldrich, St. Louis, USA) with 1% agar as described by Malec et al. (2002). The seeds were imbibed in dim white light (<10 μmol $m^{-2}s^{-1}$) delivered through a Schott NG glass filter from a white fluorescent lamp for 2 h including sterilization time.

For experiments including imbibition in darkness, the seeds were either surface-sterilized and grown on 1% MS agar as above or else sown dry on wet tissue paper. The seeds were then imbibed in total darkness for 2 h. All subsequent handling of the imbibed seeds was done in total darkness. Neither surface-sterilization nor growth conditions (tissue paper vs. MS agar) had a detectable effect on germination frequency.

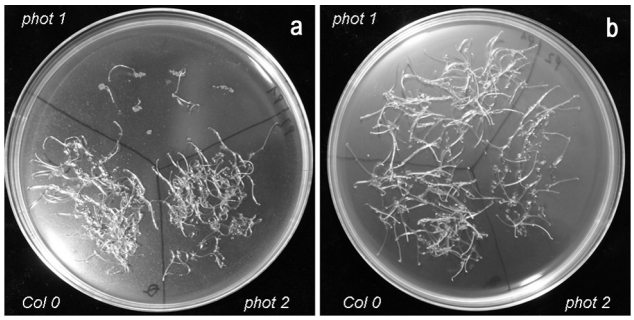

Fig. 1. Germination of *Arabidopsis* seeds imbibed for 2 h in dim light (<10 μmol $m^{-2}s^{-1}$), stratified in darkness at 4°C for 48 h and (**a**) grown at 22°C for 4 days in darkness or (**b**) irradiated with white fluorescent light (60 μmol $m^{-2}s^{-1}$) for 3 h and grown at 22°C for 4 days in darkness.

Imbibed seeds were directly subjected to a given light treatment (3 h or continuous white fluorescent light) or stratified at 4°C for 48 h in darkness and subsequently treated with light. After light treatment the seeds were grown in darkness at 22±2°C for 4 days. Germination was determined as radicle formation on the fourth day after imbibition.

STATISTICS

The experiments were done in at least three replicates (typically 50–150 seeds each) for each light treatment and each tested seed line. The data are presented as means ± standard error. Statistical significance was determined with Student's t-test at $p<0.05$. The experiments were repeated at least five times, with consistent results.

RESULTS AND DISCUSSION

During our work with *phot* mutants we noted that *phot1* seeds germinated poorly or not at all if the material was kept in darkness during or after imbibition, irrespective of stratification by chilling (Fig. 1a). They germinated normally if exposed to light for 3 h between the stratification period and four days of further growth in darkness (Fig.1b). To examine the potential effect of phototropins on the dormancy-to-germination transition we measured the germination frequencies of *phot1*, *phot2* and double *phot1phot2* mutant seeds under different light and temperature treatments.

For seeds imbibed in darkness, *phot1* and *phot2* mutants had lower dark-germination (<2%) than wild-type (WT) seeds (~15%). Stratification for 48 h at 4°C significantly stimulated the dark-germination of WT seeds to ~60% and slightly stimulated the light-independent dark-germination of *phot2*, which reached ~10%. Stratification did

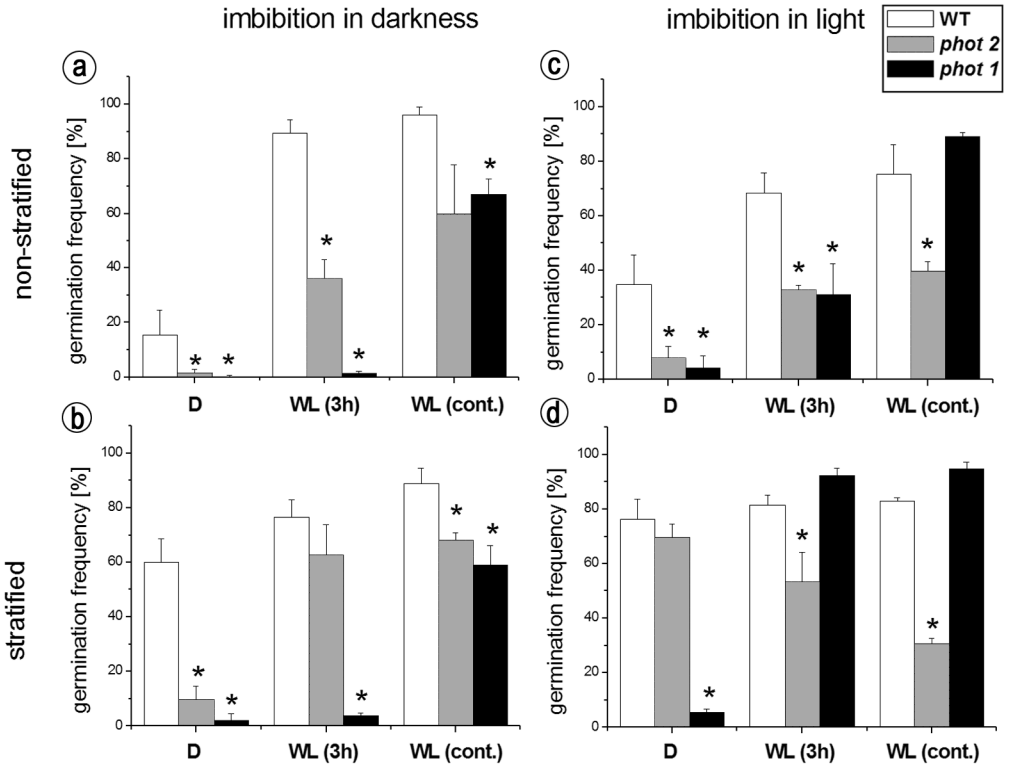

Fig. 2. Induction of germination of Col 0, *phot1* and *phot2* mutants. (**a**) Imbibition in darkness for 2 h, (**b**) Imbibition in darkness for 2 h followed by stratification for 48 h at 4°C, (**c**) Imbibition in dim light (<10 μmol $m^{-2}s^{-1}$) for 2 h; (**d**) Imbibition in dim light (<10 μmol $m^{-2}s^{-1}$) for 2 h followed by stratification for 48 h at 4°C. Seeds were grown in darkness (D) or were exposed to light (60 μmol $m^{-2}s^{-1}$) for 3 h and then grown in darkness [WL (3 h)] or under continuous light [60 μmol $m^{-2}s^{-1}$; WL (cont.)]. Germination frequency was determined on the fourth day after imbibition. *Difference between WT and mutants within treatment significant at $p<0.05$.

not stimulate dark-germination of *phot1* (Fig. 2a,b, D bars).

Irradiation of dark-imbibed seeds with white light (60 μmol $m^{-2}s^{-1}$) for 3 h prior to germination in darkness significantly enhanced the dark-germination of WT seeds to ~90%. Germination of the *phot2* mutant reached ~36%; germination of *phot1* (<2%) did not increase. When stratified seeds were irradiated as above, *phot2* germination was comparable to that of non-irradiated dark-germinated WT seeds (63%). Germination of *phot1* was below 10% under those conditions (Fig. 2a,b, WL (3 h) bars). Both *phot1* and *phot2* mutants showed lower germination frequencies under continuous light, with no stimulatory effect of stratification (Fig. 2a,b, WL (cont.) bars).

VLFR promotion of germination is saturated at very low concentrations of far-red-absorbing phytochrome form (Pfr) (Smith and Whitelam, 1990). To study the effect of VLFR stimulation on the germination of phototropin mutants, seeds were imbibed in dim light for 2 h and then germinated in darkness. In these conditions the dark-germination of WT seeds was boosted to 35%. Stratification further enhanced this effect to 76%. In contrast, *phot1* and *phot2* mutants showed less than 10% dark-germination. Stratification significantly stimulated the dark-germination of *phot2* to a level close to that of WT seeds (70%) but had no such effect on *phot1* seeds (<10% germination) (Fig. 2c,d, D bars).

When light-imbibed seeds were exposed to white light for 3 h prior to germination in darkness, germination of WT seeds roughly doubled (68%) versus dark-germinated material. Under these conditions the *phot1* and *phot2* mutants reached ~32% germination, comparable to that of dark-germinated light-imbibed WT seeds. When stratified seeds were irradiated as above, WT seed germination (82%) did not significantly differ from dark-germination of light-imbibed WT seeds (76%). Under these conditions, *phot2* seeds reached 53% germination, significantly lower than the dark-germination of light-imbibed *phot2* seeds (70%). In contrast, *phot1* exceeded 90% germination frequency under these conditions (Fig. 2c,d, WL (3 h) bars).

When light-imbibed seeds germinated under continuous white light, germination frequency

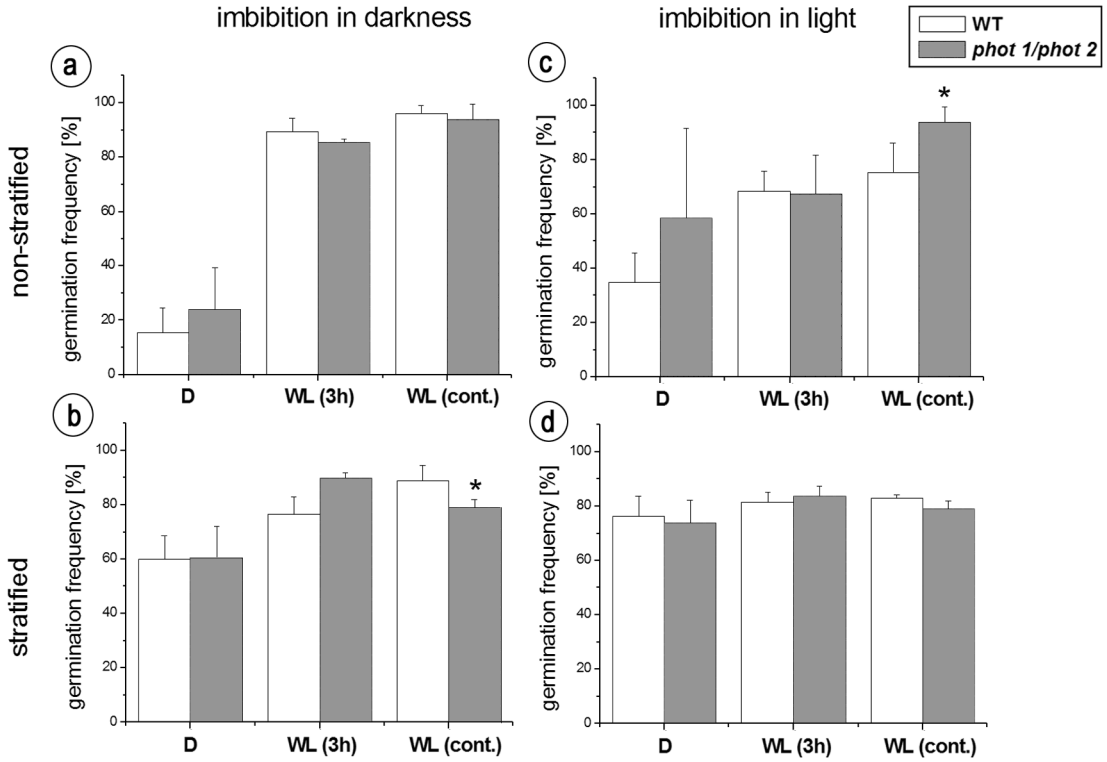

Fig. 3. Induction of germination of Col 0 and *phot1/phot2* double mutant. (**a**) Imbibition in darkness for 2 h, (**b**) Imbibition in darkness for 2 h followed by stratification for 48 h at 4°C; (**c**) Imbibition in dim light (<10 μmol $m^{-2}s^{-1}$) for 2 h, (**d**) Imbibition in dim light (<10 μmol $m^{-2}s^{-1}$) for 2 h followed by stratification for 48 h at 4°C. All experimental conditions as in Figure 2.

increased in WT (75%) and *phot1* (89%); *phot2* germination reached 40%, comparable to that of dark-germinated WT seeds. Stratification did not significantly alter germination of WT (83%) and *phot1* (94%) seeds under continuous light, and reduced germination of *phot2* seeds to 31% (Fig. 2c,d, WL (cont.) bars).

Surprisingly, our analysis of light-induced germination showed that the double *phot1phot2* mutant lacking both functional phototropins germinated at frequencies comparable to WT, irrespective of light and temperature conditions (Fig. 3a–d). *phot1phot2* seed germination in the dark equalled that of WT, and chilling boosted it markedly (Fig. 3a,b, D bars). The only statistically significant differences were noted under continuous light. In these conditions, dark-imbibed *phot1phot2* seeds had slightly lower germination than WT seeds [Fig. 3b, WL (cont.) bars], while light-imbibed non-stratified seeds of this line had slightly higher germination [Fig. 3c, WL (cont.) bars] in a manner similar to that of *phot1* seeds under these conditions. These results indicate that although functional phototropins are not essential for a photoinduction of seed germination in *Arabidopsis*, they may modulate processes involved in the dormancy-to-germination transition.

Germination of single *phot1* and single *phot2* mutants in darkness was much lower than for WT. Extensive light treatment of dark-imbibed seeds was essential for effective promotion of the germination of both single mutants. Germination of the *phot1* mutant was most effective under continuous light, but the maximum germination frequencies achieved by *phot* mutants were below the control level (WT seeds). These findings suggest that phototropins are factors modulating light-induced promotion of seed germination at low light intensities. Interestingly, a wide spectrum of light (from near UV to far-red) was found to induce VLFR-dependent germination, and the action spectrum of VLFR induction contains a broad maximum at ~400 nm (UV/blue light) (Shinomura et al., 1996). Crosstalk in photoreceptor signaling and integration of light signals with other environmental stimuli were observed in different experimental systems (Franklin et al., 2005). In *Arabidopsis*, co-action between phytochrome and phototropin systems has been demonstrated for regulation of hypocotyl growth inhibition (Folta and Spalding, 2001) and light-induced chloroplast relo-

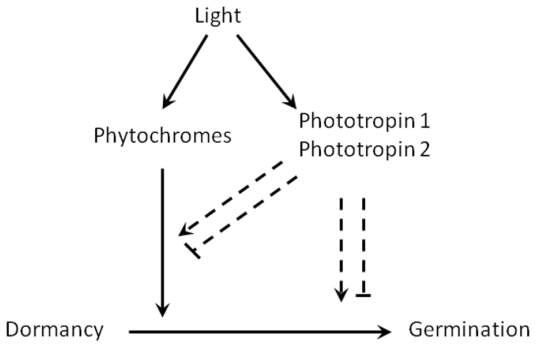

Fig. 4. Tentative scheme of the role of phototropins in control of the dormancy-to-germination transition of *Arabidopsis* seeds. Light sensed by the phototropin system may modulate the activity of a phytochrome-triggered signaling pathway and/or influence other processes (e.g., temperature-dependent) involved in promoting seed germination.

cation (DeBlasio et al., 2003; Luesse et al., 2010). Very recently the existence of light-signaling phytochrome/phototropin complexes was postulated (Jaedicke et al., 2012). Thus, the final germination response of light-stimulated seeds may depend on some unknown interactions between phytochromes and phototropins. Moreover, the decreased dark-germination frequencies of single *phot* mutants, with dark-germination of the double *phot1phot2* mutants not effected, suggest the complex character of the modulatory effect of phototropins on photoinduction of seed germination.

For light-imbibed seeds a stimulatory effect of prolonged light treatment was observed only in the *phot1* mutant. Germination of *phot1* seeds under continuous light was higher than that of WT, but *phot2* seed germination was significantly reduced. This result suggests that Phot1 may act antagonistically to Phot2 under these conditions, reducing germinability under, for example, excessive light, whereas Phot2 might be responsible for stimulating the germination of seeds exposed to direct daylight. Previously, antagonistic action of phototropins 1 and 2 was found in the phototropic response, where long-term exposure of dark-grown seedlings to light resulted in a decrease in *Phot1* transcript accumulation (Kanegae et al., 2000; Sakamoto and Briggs, 2002). On the other hand, *Phot2* transcript was found to accumulate in light-exposed seedlings (Jarillo et al., 2001; Kagawa et al., 2001). Phytochromes have been identified as photoreceptors involved in this differential gene expression (Teppermann et al., 2001; Elliott et al., 2004).

Cold-stratification did not stimulate germination of the dark-imbibed or light-imbibed seeds of the *phot1* mutant. Simultaneous treatment with both light and cold was most effective in promoting *phot1* germination. Under limiting light conditions (e.g., in dormant seeds surviving in the ground) the presence of functional Phot1 seems crucial for effective germination. In contrast, light significantly stimulated germination of dark-imbibed seeds of cold-stratified *phot2*. Also, light-imbibed *phot2* seeds germinated in darkness in a manner comparable to WT after stratification. Further light stimulation inhibited germination of these seeds. These observations suggest that the action of phototropins on seed germination is at least partially coupled with a temperature-dependent mechanism. Penfield et al. (2005) demonstrated such coupling of light and temperature signaling in seeds.

Taken together, our results show that phototropins may influence the activity of the light/cold signaling pathways involved in regulation of seed germination in *Arabidopsis*. Whereas phytochromes play a major role as triggers releasing the germination signal (Bae and Choi, 2008), we suggest that phototropins modulate the final response according to prevailing environmental conditions. In particular, the activity of the phytochrome-triggered signaling pathway and/or other processes (e.g., temperature-dependent) involved in promotion of seed germination may come under the control of the phototropin system (Fig. 4). Together with phytochromes and temperature, phototropins may be part of the signaling network controlling the dormancy-to-germination transition of *Arabidopsis* seeds and perhaps those of other physiologically dormant plant species.

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