Phytochrome E Controls Light-Induced Germination of Arabidopsis

Lars Hennig, Wendy M. Stoddart, Monika Dieterle, Garry C. Whitelam, and Eberhard Schäfer*

Institut für Biologie II, Universität Freiburg, 79104 Freiburg, Germany (L.H., M.D., E.S.); and Department of Biology, Leicester University, Leicester LE1 7RH, United Kingdom (G.C.W., W.M.S.)

Germination of Arabidopsis seeds is light dependent and under phytochrome control. Previously, phytochromes A and B and at least one additional, unspecified phytochrome were shown to be involved in this process. Here, we used a set of photoreceptor mutants to test whether phytochrome D and/or phytochrome E can control germination of Arabidopsis. The results show that only phytochromes B and E, but not phytochrome D, participate directly in red/far-red light (FR)-reversible germination. Unlike phytochromes B and D, phytochrome E did not inhibit phytochrome A-mediated germination. Surprisingly, phytochrome E was required for germination of Arabidopsis seeds in continuous FR. However, inhibition of hypocotyl elongation by FR, induction of cotyledon unfolding, and induction of agravitropic growth were not affected by loss of phytochrome E. Therefore, phytochrome E is not required per se for phytochrome A-mediated very low fluence responses and the high irradiance response. Immunoblotting revealed that the need of phytochrome E for germination in FR was not caused by altered phytochrome A levels. These results uncover a novel role of phytochrome E in plant development and demonstrate the considerable functional diversification of the closely related phytochromes B, D, and E.

In many plants, seed germination is light dependent (Casal and Sánchez, 1998). Among plant photoreceptors, only phytochromes have been shown to directly mediate induction of germination. Recently, several new insights into the molecular mechanisms of phytochrome action and its physiological role throughout the whole life cycle of plants were achieved (for review, see Whitelam and Devlin, 1997; Casal, 2000; Smith, 2000). In Arabidopsis, phytochrome is a small gene family, consisting of the five members, PHYA to PHYE (Sharrock and Quail, 1989; Clack et al., 1994). PHYB, PHYD, and PHYE are evolutionary related and clearly separated from PHYA and PHYC (Mathews and Sharrock, 1997). Studies using mutants and overexpressor lines demonstrated that individual phytochrome family members have overlapping and distinct functions (Reed et al., 1994; Smith et al., 1997). phyA and phyB are the best characterized phytochromes in Arabidopsis. phyA mediates the high irradiance response in far red light (FR; FR-HIR) and the very low fluence response (VLFR), which can also be induced by FR. In contrast, phyB mediates the red light (R)/FR-reversible low fluence response (LFR; Casal et al., 1998). Less is known about the other phytochromes. An LFR-type action of phyD could be observed in phyB seedlings (Aukerman et al., 1997; Hennig et al., 1999a). The high sequence identity of phyB and phyD (>80%) suggests very similar roles of these two phytochromes, albeit certain physiological differences have been reported (Hennig et al., 1999a). Finally, for phyE, mainly functions in the shade avoidance syndrome of adult plants have been described (Devlin et al., 1996, 1998). Furthermore, phyE has been shown to be capable of signaling to the circadian clock in seedlings (Devlin and Kay, 2000).

Light-induced germination has been studied extensively in Arabidopsis (for review, see Casal and Sánchez, 1998). This work revealed that induction of germination by R is mediated by phyB and phyA, whereas induction by FR is mediated only by phyA (Shinomura et al., 1996, 1998). Casal and coworkers demonstrated that phyA induces germination when acting in the VLFR mode. In contrast, continuous FR effectively opposes germination in many plant species, although not in Arabidopsis (Botto et al., 1996; Casal and Sánchez, 1998). Moreover, both phyB and phyD have been shown to interfere with phyA-mediated induction of germination by FR (Hennig et al., 2001). However, phyA and phyB are clearly not the only phytochromes participating in light-induced germination. R/FR-reversible induction of germination was observed in phyA phyB double mutants (Poppe and Schäfer, 1997). Nonetheless, it has remained completely unknown which other phytochromes are involved in this reaction. Here, we used additional phyD and phyE photoreceptor mutant combinations to investigate phytochrome-mediated germination in Arabidopsis in more detail.
RESULTS

Poppe and Schäfer (1997) observed R/FR-reversible induction of germination in phyA phyB double mutants. Using the original light regime of hourly pulses for 3 d, we analyzed additional photoreceptor mutants (Fig. 1). Wild-type (WT) seeds germinated efficiently in continuous white light (W) and after R pulses (30 s, 39 μmol m⁻² s⁻¹) but germinated poorly in darkness and after long wavelength FR (3 min, 35 μmol m⁻² s⁻¹) or R/FR pulses. As demonstrated before (Poppe and Schäfer, 1997), germination of phyA phyB double mutants did not differ from WT under these conditions. Likewise, phyA phyB cry1 and phyA phyB phyD triple mutants behaved in a very similar way to WT, except for less efficient germination of phyA phyB phyD in W. Moreover, phyE mutants showed an unaltered germination. In contrast, basically no light-inducible germination could be observed for seeds of phyA phyB phyE triple mutants.

Induction of germination by FR depends on phyA and is inhibited by phyB and phyD (Hennig et al., 2001). Because phyB, phyD, and phyE are evolutionarily closely related, we tested the influence of phyE on phyA-mediated germination in FR. Seeds were incubated for 24 h at 4°C in darkness; after treatment with indicated light qualities for 3 h, the seeds were stored at 25°C in darkness for 3 d. In agreement with previous reports, only R lead to high germination rates in WT and phyA under these conditions (Fig. 2). In contrast, FR and R followed by FR were as efficient as R in inducing germination of phyB. For phyD, FR and R/FR were slightly less effective than R. Seeds of the phyA phyB double mutant germinated poorly under all light conditions. Importantly, phyE seeds did not germinate appreciably after FR or R/FR and thus behaved like WT rather than phyB or phyD.

To further characterize the role of phyE in germination, we analyzed induction of germination by continuous irradiation in more detail. Seeds were incubated for 24 h at 4°C in darkness. After treatment with indicated light qualities for 3 d, seeds were stored at 25°C in darkness for another 3 d. Continuous irradiation with W, blue (B), R, or FR for 3 d induced high germination frequencies in WT (Fig. 3). In contrast, phyA seeds germinated poorly after B and not at all after FR treatment. Germination of phyB and phyD was indistinguishable from WT. Seeds of the phyA phyB phyD double mutant germinated after neither B nor FR treatment. Surprisingly, phyE seeds behaved in a very similar way to phyA; germination after B was impaired and after FR it was completely abolished. These results indicate a requirement of phyE for phyA-mediated induction of germination under continuous FR. Similarly, the phyA phyE double mutant germinated to a low extent after B and barely after FR. Finally, the phyA phyB phyE triple mutant completely failed to show light-inducible germination under these conditions.

The requirement of phyE on germination in FR might be caused by altered phyA levels in phyE seeds. To test this hypothesis, we analyzed total phyA amounts by immunoblotting. Total protein...
End, seedlings were exposed to hourly FR pulses required for other phyA-mediated processes. To this end, seedlings were exposed to hourly FR pulses (36 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) or continuous FR (3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). After 5 d hypocotyl lengths were measured. The results (Fig. 7A) show that WT and phyE seedlings did not differ either under FR pulses or under continuous FR. In contrast, phyA seedlings remained elongated under both conditions. Interaction of photoreceptors can be observed more easily under nonsaturating conditions (Hennig et al., 2001). Consequently, seedlings of WT, phyA, phyB, phyD, and phyE were grown under nonsaturating continuous FR (0.6 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). WT seedlings showed an inhibition of elongation of about 40% under these conditions (Fig. 7B). In contrast, growth of phyB mutants was inhibited by >50%. Nonetheless, neither phyD nor phyE differed from the WT.

**DISCUSSION**

Induction of germination by light is controlled by phyA and phyB (Shinomura et al., 1996). However, in addition to these two photoreceptors other pigments have been shown to be involved in this process. R/FR-reversible germination of phyA phyB double mutants led to the conclusion that at least one other member of the phytochrome family controls germination in Arabidopsis (Poppe and Schäfer, 1997). Moreover, no reports about a possible involvement of other photoreceptors, e.g. cryptochromes, exist. To determine the role of additional phytochromes in germination, we used several photoreceptor single, double, and triple mutant combinations.

**phyE Controls R/FR-Reversible Germination in phyA phyB**

A program of hourly light pulses applied for 3 d induced R/FR-reversible germination in phyA phyB double mutants. Both, phyA phyB phyD and phyA phyB cry1 triple mutants displayed the same pattern of germination. Therefore, neither phyD nor cry1 are required for germination under these conditions. Given the high sequence conservation of phyB and phyD, it was surprising that absence of phyD did not further impair R-induced germination. Under none of the diverse conditions tested was any direct demonstration of germination control by phyD obtained. In contrast, phyA phyB phyE triple mutants were severely impaired in light-induced germination and lacked any R/FR-reversible germination. We conclude, therefore, that phyE plays an active role in
germination of Arabidopsis. If any additional photoreceptors were involved in this process, they clearly depend on the presence of at least one of phyA, phyB, or phyE. Moreover, the R/FR-reversible nature of induction of germination by phyE implies that this phytochrome can function in the LFR mode of phytochrome action.

The similar actions of phyB and phyE in germination prompted us to investigate negative effects of these phytochromes on phyA action. Previously, we described inhibition of phyA-mediated germination by phyB and phyD (Hennig et al., 2001). However, we were not able to detect any negative interference of phyE with phyA function. This difference could be due to different expression levels of phyB, phyD, and phyE. Alternatively, it could reflect inherent mechanistic differences in the biological actions of these phytochromes.

**phyE Is Required for Germination in Continuous FR but Not for Other phyA Responses**

In addition to its involvement in germination after pulsed irradiations, phyE proved to affect germination in continuous light. Surprisingly, phyE was required for induction of germination by continuous FR. This observation was not expected, because phyA is generally regarded as being both necessary and sufficient for effects of FR. Responses of phyA to FR are usually attributed to either the VLFR or the HIR mode of phytochrome action (Casal et al., 1998). We analyzed the consequences of phyE deficiency on additional responses of either the VLFR or HIR type. None of three other VLFR reactions (namely, induction of cotyledon opening, interference with gravitropism, and growth inhibition by FR pulses) was altered in phyE mutants. Further experiments showed that phyE was also not required for control of hypocotyl growth under continuous FR involving an HIR. However, phyE also had no negative influence on inhibition of hypocotyl elongation by intermediate fluence rates of FR. In this respect, phyE behaves like phyD and not like phyB, which counteracts the FR-HIR (Hennig et al., 1999b). Therefore, only germination under FR, but not all phyA-mediated responses to FR, depend on the presence of phyE.

Immunoblots showed that the amount of phyA in seeds is not influenced by the presence or absence of phyE. Consequently, the failure of phyE seeds to germinate under FR is not caused by reduced phyA levels. Several other mechanisms could account for the behavior of the phyE mutant. phyE might control the level of signaling intermediates required by phyA for inducing germination. Alternatively, phyE might act as an additional photoreceptor for FR. In this regard, it is noteworthy that a recent report described profound differences between the photochemical properties of phyB and phyE (Eichenberg et al., 2000). Further investigations will be required to resolve this issue.

**The Physiological Roles of phyB, phyD, and phyE Differ Considerably**

The three closely related photoreceptors phyB, phyD, and phyE have been shown to be involved in responses to R/FR (Devlin et al., 1998, 1999). All three phyB-like phytochromes can function in the LFR mode of phytochrome action. Moreover, they are all capable of signaling to the circadian clock (Devlin and Kay, 2000). For phyB and phyD, but not for phyE, an involvement in control of hypocotyl elongation has been shown (Reed et al., 1993; Aukerman et al., 1997; Hennig et al., 1999a). Here, we report that phyB and phyE, but not phyD, participate in R/FR-reversible germination. Poppe and Schäfer (1997) demonstrated that the photobiology of induction of germination by phyE, termed phyX by these authors, differed greatly from that of phyB: Requirement of prolonged irradiation and fluence rate re-
response curves were more similar to an HIR than to the classical LFR. Likewise, the loss-of-reversibility kinetics were much faster for the phyE than for the phyB response (Poppe and Schafer, 1997). Our results show that phyB and phyE also have opposing effects. Whereas phyB exerts an inhibitory action on a diverse set of phyA functions under FR, including germination (Hennig et al., 2001), phyE is required for germination under FR. Taken together, phyB is more similar to phyD than to phyE regarding interference with phyA-mediated germination. In contrast, phyB is more similar to phyE than to phyD regarding positive control of germination after R pulses. Hence, the physiological functions of the closely related phyB, phyD, and phyE are only partly redundant but differ in several aspects.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Light Sources

The Landsberg erecta (Ler) Heynh. was used (obtained from Lehle Seeds, Tuscon, AZ). The mutants were phyA-201 (Nagatani et al., 1993; Reed et al., 1994), phyB-5 (Koornneef et al., 1980; Reed et al., 1993), phyD-1 (Aukerman et al., 1997), phyE-1 (Devlin et al., 1998), and hy4-2.23 (containing a defect CRY1 gene, Koornneef et al., 1980; Ahmad and Cashmore, 1993). Double and triple mutants were generated by crossing (Devlin et al., 1998, 1999; Hennig et al., 1999a).

Seeds were plated on four layers of water-soaked filter paper, which were placed into clear plastic boxes. A 24-h dark treatment at 4°C was followed by induction of germination by the light quality and duration indicated in Figures 1 to 4 and 7B. Subsequently, samples were further incubated in the dark at 25°C. Standard B (436 nm, 35 μmol m⁻² s⁻¹), R (656 nm, 30 μmol m⁻² s⁻¹), or FR (730 nm, 20 μmol m⁻² s⁻¹) fields were used. Repetitive pulse irradiation with monochromatic light was achieved using computer-controlled Leitz Prado light projectors (Leitz, Wetzlar, Germany) with 660 nm of DAL interference filters or RG9 color glasses (Schott, Mainz, Germany). Alternatively, seeds were sown onto plates (0.8% agar Lehle medium, Lehle Seeds) and chilled at 4°C for 3 d. Germination was induced by 30 min of white fluorescent light (80 μmol m⁻² s⁻¹), and plates were incubated in the dark at 22°C for 24 h, after which irradiation was started (Figs. 5, 6, and 7A).

Protein Extraction and Immunoblotting

Seeds were ground in liquid nitrogen and extracted with preheated SDS sample buffer (65 mM Tris-HCl, pH 6.8, 4 M urea, 3% [w/v] SDS, 10% [v/v] glycerol, 10 mM dithioerythritol, 0.05% [w/v] bromphenol blue). After heating to 95°C for 5 min, the crude extracts were clarified by centrifugation at 15 min at 20,000g (25°C).

SDS-PAGE, protein blotting, and immunodetection were performed as described by Harter et al. (1993) using CSPD-STAR (New England Biolabs, Beverly, MA) as the substrate for alkaline phosphatase-conjugated secondary antibodies. Monoclonal antibodies against phyA of Arabidopsis were a...
Determination of Hypocotyl Length and Germination Frequencies

Hypocotyl lengths were measured manually for at least 20 seedlings. The mean value and se from at least three independent experiments (Fig. 7B) or the results of one representative experiment are shown (Fig. 7A). Significant differences of hypocotyl lengths of dark-grown seedlings were not observed (data not shown). Germination percentages of 80 to 120 seeds were determined by taking the protrusion of the radicle as the criterion of germination. The mean value and se of at least four independent experiments are shown. At least two independent seed batches per mutant were used for germination experiments.

Determination of Hypocotyl Growth Orientation

Seeds were sown onto plates (1.2% agar Lehle medium, Lehle Seeds) and chilled at 4°C for 3 d. Germination was induced by 30 min of white fluorescent light (80 µmol m⁻² s⁻¹), and plates were orientated vertically and incubated in the dark at 22°C for 24 h, after which irradiation was started. Plates were maintained at a vertical orientation throughout. After 3 d of treatments, plates were photographed, and measurements were taken of at least 60 (dark control) or 100 seedlings. The angles of hypocotyls relative to vertical were recorded, resulting in values from 0 (vertical) to ±180 degrees.

ACKNOWLEDGMENTS

We thank Peter H. Quail for the antiserum against phyA. We thank Dr. Trish Ingles for help with the experiments on growth orientation. Furthermore, we thank Rena Wiehe for excellent technical assistance and Claudia Büche for critical reading of the manuscript.

Received June 25, 2001; returned for revision July 28, 2001; accepted August 12, 2001.

LITERATURE CITED


