Seed Germination of *Arabidopsis thaliana* phyA/phyB Double Mutants Is under Phytochrome Control

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We examined the photocontrol of seed germination in the phyA/phyB double mutants of *Arabidopsis thaliana* seeds. Dormant phyA/phyB seeds showed a red/far-red light (R/FR)-reversible induction of seed germination. This suggests the involvement of at least one other phytochrome, phyC, phyD, and/or phyE, in controlling seed germination. We designated this spectrally active phytochrome in the phyA/phyB double mutant phyX. The full reversibility of the R-induced germination by subsequent FR pulses, and the observation that the response is reversible by FR, even after a 3-h R treatment, indicates that this phyX response belongs to the low-fluence-response type. Thus, this phyX response is functionally related to phyB-mediated responses. However, in contrast to phyB-controlled seed germination, this phyX-mediated response needs a prolonged imbibition period and exhibits reversibility kinetics different from that needed for phyB. Furthermore, this phyX response requires a prolonged irradiation time and shows a fluence rate response dependency, showing a similarity to the high irradiance response of photomorphogenesis. Thus, phyX, with regard to its control of seed germination, is a functionally new phytochrome that shares some characteristics of both phyA- and phyB-mediated responses.

Plants as sessile organisms have developed an enormous capacity to adapt to changes in the natural environment, in which light is probably the most critical variable. Plants have evolved a series of photoreceptors to monitor quality, quantity, and spatial and temporal patterns of light (Kendrick and Kronenberg, 1994). The most prominent photoreceptors are phytochromes, blue light, UV-A, and UV-B receptors.

Photoregulation occurs throughout the entire life cycle of the plant, starting from seed germination and continuing through seedling de-etiolation, shade avoidance, and flowering in adult plants (Kendrick and Kronenberg, 1994). Photocontrol of seed germination was one of the very first R/FR-reversible responses observed (Borthwick et al., 1952) and led to the discovery of phytochrome by Butler et al. (1959). Physiological, spectroscopical, and biochemical data indicated the presence of different types of phytochromes (Furuya and Schäfer, 1996). Conclusive evidence for the presence of distinct types of phytochromes was provided by cloning and sequencing five genes of the Arabidopsis genome (Sharrock and Quail, 1989; Clack et al., 1994). Genetic engineering approaches (Quail et al., 1995), defined photoreceptor mutants (Furuya, 1993), and microinjection in chromophore-deficient mutants (Neuhaus et al., 1993; Kunkel et al., 1996) have been used to analyze the different roles of these phytochromes in controlling photomorphogenic responses. Most of the research on this topic has focused on the model plant *Arabidopsis thaliana* because it is easily transformed and mutants are available in which phyA, phyB, or both are absent.

As early as 35 years ago, Shropshire et al. (1961) performed an action spectrum for seed germination of Arabidopsis that clearly demonstrated phytochrome involvement in this response. More recently, Shinomura et al. (1994) demonstrated the primary role of phyB in the induction of seed germination of Arabidopsis. They also showed that phyA can only induce germination after a prolonged imbibition. Detailed action spectra for seed germination performed in wild-type, phyA, and phyB mutants revealed a typical R/FR-reversible LFR mediated by phyB, whereas the germination response mediated by phyA turned out be a VLFR with a 104-fold higher sensitivity to light (Shinomura et al., 1996). The phyA-mediated VLFR was detectable after 48 h of imbibition, which allowed phyA accumulation, whereas the phyB-mediated LFR was already observable 3 h after imbibition. However, these authors did not find any indication for the role of other phytochromes controlling seed germination, prompting these authors to conclude that only phyA and phyB control seed germination (Shinomura et al., 1996).

It was observed in several laboratories that phyA/phyB double mutants show a light-dependent germination. For example, Yang et al. (1995), analyzing the effects of GAs on the control of seed germination, not only reported that phyA/phyB seeds germinate to 95% in continuous WL but also observed a slight, inducible effect of seed germination after a single R pulse and a difference in the effectiveness of treatments with GAs after R or FR pulses (Yang et al., 1995). Carabelli et al. (1996) demonstrated an R/FR-reversible induction of the abundance of the A-th2 homeobox gene transcripts even in phyA/phyB double mutants, indicating an involvement of phyX in this specific response. Devlin et al. (1996) observed an R-reversible, early-flowering response and also the promotion of internode elongation after end-of-day FR treatments in the phyA/phyB double mutant.

Abbreviations: FR, far-red light; LFR, low-fluence response; phyA (or B), spectrally active phytochrome A (or B); phyX, spectrally active phytochrome in the phyA/phyB double mutant; R, red light; VLFR, very-low-fluence response; WL, white light.
Here we address the question of whether photocontrol of seed germination can be described by a phytochrome other than phyA or phyB. We used seed germination as the assay, and we describe evidence supporting a role for phyX in controlling seed germination.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Measurement

We used the following ecotypes and null mutants of Arabidopsis thaliana (L.) Heyn.: Landsberg erecta (Ler) (obtained from Lehle Seeds, Tuscon, AZ), phyA-201 (Nagatani et al., 1993), phyB-1 and phyB-5 (Koornneef et al., 1980; Reed et al., 1993); and phyA-201/phyB-5 (Reed et al., 1994). All of the seeds that were used derived from plants grown under continuous WL in temperature-controlled (21°C) rooms. Since we could not detect the same light-induced germination in freshly produced phyA/phyB seeds (data not shown), for the experiments presented here we used seeds that were stored dry for more than 6 months at 4°C. Seeds were plated on four layers of water-soaked filter papers, which were placed into clear plastic boxes. The standard sowing procedure in normal WL was followed by a 1-h FR treatment and a 24-h dark-cold treatment at 4°C prior to the irradiations of the seeds. After irradiation the plants were incubated in the dark at 25°C. The maximum germination rate for the batches was determined to be 99% in continuation and a 24-h dark-cold treatment at 4°C prior to the irradiations. The germination percentages were determined for each population after 6 d (Fig. 1A). The minor dark germination rate could be reversed in the wild type, pkyA-201, and phyA-201/phyB-5 to absolute zero by a 1-h FR treatment directly after sowing. The dark germination rate of phyB-1 was not influenced by this FR irradiation (data not shown). Strong induction of germination (75–95%) could be observed in wild-type, phyA, and phyB seeds after a 3-h R treatment, whereas a 20% germination rate was observed in the phyA/phyB double mutant under the same conditions. A 3-h FR treatment did not induce significant germination in phyA and phyA/phyB, confirming the role of phyA in FR-mediated responses, as previously shown (Shinomura et al., 1994). The 20% germination of phyA/phyB double mutants after 3 h of R was fully reversible by a subsequent 3-h FR treatment, indicating the involvement of another R/FR-reversible system controlling seed germination in phyA/phyB double mutants, which we designate here as phyX. Stronger induction of seed germination for

Light Sources

Modified Prado 500-W universal projectors (Leitz, Wetzlar, Germany) were used as light sources for repetitive pulse

Figure 1. Germination of wild-type (L.e.), phyB-1, phyA-201, and phyA-201/phyB-5 seeds of A. thaliana after either single light treatments (A) or repetitive pulse treatments (B). A, Dormant seeds were sown on water-soaked filter papers, cold-treated for 24 h at 4°C and either kept in the dark or irradiated by R (3 h, 3 W/m² = 16.5 μmol m⁻² s⁻¹), FR (3 h, 2.6 W/m² = 15.5 μmol m⁻² s⁻¹), or 3 h of R followed by 3 h of FR. After the irradiation the seeds were kept in the dark for 5 to 6 d until the measurement. B, Dormant seeds were sown on water-soaked filter papers, cold-treated for 24 h at 4°C, and irradiated for 72 h with repetitive, hourly light pulses. The repetitive light programs were as follows: 30 s of R/1 h of dark or 3 min of FR/1 h of dark or 30 s of R/3 min of FR/1 h of dark. After the 72-h irradiation program the seeds were kept for another 3 d in the dark until the measurement. Values are means ± se from at least five independent experiments.
phyA/phyB double mutants was obtained by repetitive light pulses. Seeds were irradiated hourly with 30 s of R, 3 min of FR, or 30 s of R followed by 3 min of FR for 3 d. The germination percentages were scored after an additional 3 d of dark incubation following the light programs (Fig. 1B). The results obtained for the wild-type, phyA, and phyB mutants were very similar to those observed after a single light treatment (Fig. 1A).

The data shown in Figure 1 support a role for at least three phytochromes in seed germination. The lack of a FR induction of seed germination and the complete photo-reversibility in the phyA mutant indicates an LFR mediated by phyB (Fig. 1B). The strong induction by FR in the phyB mutants and, therefore, the absence of reversibility is an indication of a phyA-mediated VLFR. The different germination rates of wild-type and phyB mutants after FR or R/FR light treatments can be explained by an antagonistic influence of phyB under these irradiation conditions. It is interesting that multiple R pulses increased the germination rate in the phyA/phyB double mutant compared with a single 3-h R irradiation. This stronger induction was fully reversible by subsequent FR treatments, illustrating the involvement of another R/FR-reversible system. With respect to the complete FR reversibility and the lack of an FR induction of germination in phyA-phyB-5, phyX shares with phyB the LFR mechanism. No significant difference could be detected if the R was substituted with WL in these experiments (data not shown).

Light-Controlled Seed Germination in phyA/phyB Is Dependent on the Preincubation Time

We addressed the question of whether phyX shares similarity to either phyA or phyB by examining the imbibition time dependency of seed germination in phyA, phyB, and phyA/phyB.

Shinomura et al. (1994, 1996) reported that phyB control of seed germination is already detectable immediately after the imbibition of the seeds, whereas phyA control of germination needs a prolonged dark incubation. Figure 2 shows that seed germination in the phyA/phyB double mutant has a dark imbibition time dependency that is qualitatively similar to the phyB mutant. A 20-h dark incubation is necessary to obtain a significant light-induced germination in phyA/phyB, although there is clearly a quantitative difference in the percentage of germination between phyB and phyA/phyB after a 3-h WL treatment. This indicates that the capacity of phyA/phyB seeds to respond to light develops during a dark preincubation. With respect to the imbibition time dependency, phyX shows a reaction that is more closely related to phyA than to phyB-mediated seed germination. There was no significant difference between a preincubation at 4 or 25°C (data not shown).

Fluence Rate and Irradiation Time Dependence of phyX-Mediated Seed Germination

To gain information about the fluence requirement for phyX-mediated induction of seed germination, fluence rate-response curves for 1- to 72-h irradiation periods of WL were analyzed with phyA/phyB double mutants. phyA/phyB seeds were preincubated for 24 h in darkness and irradiated for 1 to 72 h with WL at fluence rates ranging from 0.5 mW/m² to 50 W/m². Figure 3A shows that irradiation periods of 1 h led even at the highest fluence rates to marginal inductions of seed germination. With increasing irradiation times and with increasing fluence rates, a higher germination rate could be observed. These data clearly show a fluence rate and a time dependence of the light induction of seed germination in phyA/phyB. Since reciprocity does not hold, this response can be classified as a high-irradiance response. Figure 3B shows a comparison among the germination rates of phyB-5, phyA-201, and phyA-201/phyB-5 after a 3-h WL irradiation of different fluence rates. Both phyA and phyB mediate the germination response much more efficiently than phyX under these conditions.

Loss of Reversibility in phyA and phyA/phyB

We have shown that the light-induced germination responses mediated either by phyB or phyX are reversible by subsequent FR pulses (Fig. 1). This reversibility is lost if an increasing dark incubation is applied between the R and FR irradiation. Figure 4 illustrates the differences in the time courses of the loss of FR reversibility of R-induced germination in phyA and phyA/phyB. Both mutants are completely reversible if 1 h of FR is applied directly after 3 h of R irradiation. An increasing dark-incubation time between these two light treatments goes along with a slowly increasing loss of reversibility in both of the mutants. For approximately 8 h after the 3-h R treatment, both phyA and phyA/phyB double mutants show the same slope in the loss-of-reversibility pattern (Fig. 4A). In phyA a further increase in the dark-incubation time before the reverting FR pulse leads to an enormous loss of reversibility, whereas the phyA/phyB double mutant has already lost more than 50% of its reversibility at this time. A 24-h dark incubation before the FR irradiation...
DISCUSSION

Another Phytochrome (phyX) Controls Seed Germination in phyA/phyB Seeds

Specific functions of phyA and phyB controlling light-induced Arabidopsis seed germination have recently been described (Shinomura et al., 1994, 1996; Botto et al., 1995, 1996).

phyB controls seed germination in a R/FR-reversible manner (LFR), whereas phyA shows a VLFR. Shinomura et al. (1996) concluded that seed germination is regulated by phyA and phyB but not by other phytochromes. However, the analysis of light-dependent seed germination in phyA/phyB double mutants showed for repetitive light pulses a classic R/FR-reversible induction of seed germination (Fig. 1B). These data clearly show the involvement of another R/FR-reversible photoreceptor system (probably phyC, D, and/or E). FR does not induce seed germination in phyA/phyB mutants, either by a single or by a repetitive light treatment. Also, the light induction of seed germination is fully reversible by a subsequent FR treatment. Both phenomena indicate that the phyX-mediated response is an LFR type.

In wild-type seeds the induction by FR was less than that in phyB seeds. This observation was previously described.

Figure 3. Effects of WL irradiation time and fluence rate on the germination of phyA-201/phyB-5 seeds. A, Dormant seeds of phyA-201/phyB-5 were sown on water-soaked filter papers, cold-treated for 24 h at 4°C, and irradiated for different times with different fluence rates of WL. Values are means from at least three independent experiments. The se of each value, which is not shown to gain more clearance in this diagram, is always in the range of the values in Figures 1, 2, and 4. B, Dormant seeds of phyA-201/phyB-5, phyA-201, and phyB-5 were sown on water-soaked filter papers, cold-treated for 24 h at 4°C, and irradiated for 3 h with different fluence rates of WL. After the irradiation the seeds were kept in the dark for 5 to 6 d until the measurement. Values are means ± se from at least three independent experiments.

leads to full loss of reversibility of the induction in both mutants. In Figure 4, B shows the same data as A, but it is plotted in a loss-of-reversibility diagram, showing the percentages of the reversibility that are lost during the increasing dark-incubation time between the R and FR treatment. This graph emphasizes the differences between the loss of reversibility in phyA and phyA/phyB, since the curve of phyA/phyB is monophasic, whereas the curve of phyA is biphasic. With respect to reversibility, the phyX-mediated germination response in phyA/phyB shows a different FR-reversibility pattern than the phyB-mediated response in phyA.

Figure 4. Effects of an increasing dark-incubation time between 3 h of R treatment (3 W/m² = 16.5 μmol m⁻² s⁻¹) and 1 h of FR irradiation (2.6 W/m² = 15.5 μmol m⁻² s⁻¹) on the germination rate of phyA-201 and phyA-201/phyB-5. Dormant seeds were sown on water-soaked filter papers, cold-treated for 24 h at 4°C, and irradiated with 3 h of R. Different times of dark incubation after this R treatment were followed by a 1-h FR irradiation. After the irradiation the seeds were kept in the dark for 5 to 6 d until the measurement. Values are means ± se from at least three independent experiments. A, The germination rate is scored. B, The same data as in A are shown in a loss-of-reversibility diagram.
by Shinomura et al. (1994) and Reed et al. (1994). It was interpreted as an inhibition of phyA-mediated responses by phyB in the R-absorbing form. This effect seems to be even more pronounced when the inhibition of hypocotyl growth is analyzed in phyB-overexpressing seedlings (Wagner et al., 1996).

**Photocontrol by phyX Depends on Preincubation Time, Fluence Rate, and Duration of Irradiation**

Whereas responsiveness of Arabidopsis seed germination to phyB is present immediately after imbibition, responsiveness to phyA needs a prolonged preincubation time. This difference in sensitivity parallels the accumulation of newly synthesized phyA (Shinomura et al., 1994). Thus, the capacity to respond to phyA seems to be primarily controlled by the amount of phyA molecules present and not by changes in the signal transduction. The dependence of the preincubation in phyA/phyB seeds (Fig. 2) parallels the dependence in phyB seeds. Unfortunately, there are no data available describing the relative amounts of phyC, D, and E during the imbibition. Therefore, three possibilities can be proposed to explain the fluence rate and time dependence of seed germination in phyA/phyB: Either the kinetics of accumulation of phyX are the same as the kinetics of phyA or a developmental dependence to respond to phyX or a combination of both is causing the dependence of light-regulated seed germination of phyA/phyB on the imbibition period. The answer to the question concerning which phytochrome(s) mediate the induction of seed germination in phyA/phyB has to wait until appropriate triple mutants (e.g. phyA/phyB/phyC) are available.

Maximal germination can be induced via phyA and phyB by a single light pulse (Shinomura et al., 1996), but for the phyX-mediated response even a fluence rate of 43 W/m² is insufficient to induce germination if the irradiation time is 1 h or less. Since photoequilibrium of phyX is expected to be established, we conclude that phyX function requires a strong time-dependent component (Fig. 3). In addition to a time dependency, the response is also fluence rate-dependent, thus showing characteristics of a high-irradiance response (Hartmann, 1966). The lack of the inducibility of germination of the double mutant by short light pulses makes the analysis of the reversibility pattern more difficult than in the phyA mutants. phyA-201 shows the same reaction pattern in the reversibility assay (Fig. 4), when the light pulses are reduced to 10 min of R or 10 min of FR, respectively, whereas the germination of phyA/phyB is not significantly induced under these conditions (data not shown). A prolonged induction of more than 10 h of irradiation leads in both mutants to only an incomplete reversion of the germination by subsequent FR pulses. The germination after irradiation is induced in phyA by phyB and phyX together. Figure 4A shows that the signal transduction starting from phyB with respect to seed germination is rather slow, since it can be completely reversed for about 8 h after the inducing R treatment, whereas the signal coming from phyX starts decreasing its reversibility already 1 h after the R pulse. We assume that the minor loss of reversibility in phyA during the first 8 h of dark incubation after the R treatment is due to the loss of reversibility of phyX-mediated germination.

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