GIGANTEA Regulates Phytochrome A-Mediated Photomorphogenesis Independently of Its Role in the Circadian Clock[1][W][OA]

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GIGANTEA (GI) is a nuclear protein involved in the promotion of flowering by long days, in light input to the circadian clock, and in seedling photomorphogenesis under continuous red light but not far-red light (FR). Here, we report that in Arabidopsis (*Arabidopsis thaliana*) different alleles of *gi* have defects in the hypocotyl-growth and cotyledon-unfolding responses to hourly pulses of FR, a treatment perceived by phytochrome A (phyA). This phenotype is rescued by overexpression of GI. The very-low-fluence response of seed germination was also reduced in *gi*. Since the circadian clock modulates many light responses, we investigated whether these *gi* phenotypes were due to alterations in the circadian system or light signaling per se. In experiments where FR pulses were given to dark-incubated seeds or seedlings at different times of the day, *gi* showed reduced seed germination, cotyledon unfolding, and activity of a luciferase reporter fused to the promoter of a chlorophyll *a/b*-binding protein gene; however, rhythmic sensitivity was normal in these plants. We conclude that while GI does not affect the high-irradiance responses of phyA, it does affect phyA-mediated very-low-fluence responses via mechanisms that do not obviously involve its circadian functions.

Phytochromes are plant photoreceptors with two interconvertible forms: Pr and Pfr (Chen et al., 2004). The molecule is synthesized in the Pr form, which absorbs maximally in red light (R), and becomes phototransformed to Pfr, with maximum absorbance in far-red light (FR). The absorption spectra of these forms show significant overlap, and, therefore, exposure of dark-grown seedlings to FR establishes a small amount of Pfr and R is unable to drive all phytochrome to the Pfr form. Higher plants possess several phytochromes encoded by divergent genes (*PHYA* through *PHYE* in Arabidopsis [*Arabidopsis thaliana*]; Quail et al., 1995). Phytochrome A (phyA) mediates two photobiologically distinct types of response: the very-low-fluence response (VLFR) and the high-irradiance response (HIR; Casal et al., 2003). Some tissues are so sensitive to phyA that even a few molecules in the Pfr form induce a response (Furuya and Schäfer, 1996). Since this effect can be saturated by a brief pulse of dim radiation of any wavelength between 300 and 780 nm (Shinomura et al., 1996), the response is called VLFR. A typical VLFR is the induction of germination in Arabidopsis seeds (Botto et al., l996; Shinomura et al., 1996). A brief pulse of FR is enough to induce germination of sensitized seeds, but it has negligible effects on the length of the hypocotyl or the unfolding of the cotyledons in etiolated seedlings. The latter responses require prolonged exposures to FR to become effective and are therefore called HIR. Seed germination and hypocotyl growth are different physiological processes, but the differences between VLFR and HIR are not simply the consequence of the different kinetics of such physiological processes. In the case of hypocotyl growth, the HIR cannot be equated to the sum of multiple VLFRs. If the pulses of FR are repeated with different frequencies, hypocotyl-growth inhibition increases gradually between 297 and 157 min of dark interval between the 3-min FR pulses (Casal et al., 2000). Increasing pulse frequency from one every 157 min to one every 27 min has no additional effect, describing a plateau that defines the contribution of the VLFR to long-term inhibition of hypocotyl growth. This plateau, however,

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represents a relatively small proportion of the effect that can be reached with continuous FR, indicating that the HIR is much more than the additive effect of repeated VLFRs. It is only when pulse frequency increases beyond one 3-min pulse every 27 min that a second component of the response, the true HIR, becomes evident. Very frequent pulses of FR are required to obtain the same effect as under continuous FR (Casal et al., 2000; Shinomura et al., 2000). Mutations at the PAS2 motif of the C-terminal domain of phyA eliminate the HIR but not the VLFR (Yanovsky et al., 2002). The *fhy3* mutant retains VLFR but shows severely impaired HIR (Yanovsky et al., 2000). The HIR of the *Lhcb1*2* gene requires a region of the promoter that is fully dispensable for the VLFR (Cerdán et al., 2000). Thus, VLFR and HIR can be dissected not only in photobiological experiments but also by means of genetic and molecular tools. There are mutants lacking the HIR and not the VLFR, but no mutant with reduced VLFR and normal HIR has been identified. The accessions Columbia (Col) and Nossen have reduced VLFR compared to Landsberg *erecta* (L*er*), but this could be the result of loss-of-function alleles involved in the repression of VLFR in L*er* rather than loss-of-function alleles in the other accessions (Yanovsky et al., 1997; Alconada-Magliano et al., 2005). Cryptochrome 2, in particular the allele of the cryptochrome 2 gene present in the accession Cape Verde, enhances phyA-mediated VLFR of cotyledon unfolding (Botto et al., 2003) and phytochrome E enhances the VLFR of seed germination (Hennig et al., 2002). However, no downstream component with a general effect on VLFR has been identified. Here, we describe GIGANTEA (GI) as a positive regulator of VLFR mediated by phyA. GI is a nuclear protein of unknown biochemical function that regulates flowering, circadian rhythms, hypocotyl growth under continuous R (presumably due to a defect in phyB signaling) and blue light, starch accumulation, and resistance to stress (Eimert et al., 1995; Kurepa et al., 1998; Fowler et al., 1999; Park et al., 1999; Huq et al., 2000; Tseng et al., 2004; Mizoguchi et al., 2005; Paltiel et al., 2006; Martin-Tryon et al., 2007).

RESULTS

We first examined the role of GI on plant growth in different light conditions and in the dark. The *gi* mutant showed no obvious morphological phenotype in darkness. The length of the hypocotyl was similar to the wild type (e.g. Col wild type = 16 ± 0.3 mm; *gi-2* = 16 ± 0.2 mm), and the cotyledons remained closed and unexpanded. Under hourly pulses of either FR or R, cotyledon-unfolding and hypocotyl-growth responses were deficient in six different alleles of *gi* compared to their respective wild types (Fig. 1). Ectopic expression of *GI* in *GI OX gi-11* and *GI OX gi-2* transgenics (David et al., 2006; Gould et al., 2006) enhanced cotyledon-unfolding and hypocotyl-growth responses to hourly pulses of either FR or R compared to the *gi-11* or *gi-2* mutant (Fig. 1). The responses of *GI OX gi-11* and *GI OX gi-2* were at least as large as the wild-type responses, indicating that the wild-type allele fully rescues the mutant phenotype.

The seedlings were also exposed to hourly R/FR pulses predicted to establish a series of calculated proportions of phytochrome in the FR-absorbing form (Pfr/P). The *phyA* mutant failed to respond to the lowest calculated Pfr/P, which corresponded to the VLFR, but it responded to higher Pfr/P, which corresponded to the phyB-mediated response (Yanovsky et al., 1997; Supplemental Fig. S1). The *phyA gi* double mutant behaved like *phyA* in the VLFR range mutation. *phyA* was not epistatic to *gi* under pulses providing higher Pfr/P (Supplemental Fig. S1), suggesting a positive role of GI in phyB-mediated responses consistent with previous proposals (Huq et al., 2000).

Under continuous FR *gi* showed weaker de-etiolation than the wild type only at the lowest fluence rates tested here, but this effect decreased at higher fluence rates (Supplemental Fig. S2). The *gi* phenotype at these lowest fluence rates is likely due to the reduction in VLFR in these mutants; the reduction of the phenotype

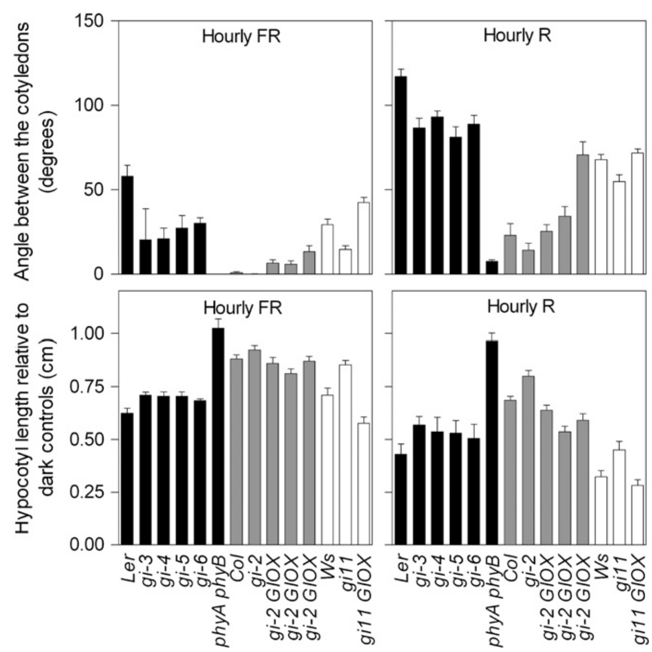

Figure 1. The effects of hourly pulses of FR or hourly pulses of R on cotyledon unfolding and hypocotyl-growth inhibition are reduced in different *gi* mutant alleles compared to the wild type and rescued by ectopic expression of *GI*. One-day-old seedlings were transferred to the indicated light treatments for 3 d before measurements. The *phyA phyB* mutant is included for comparative purposes. Data are means and SE of at least five replicate boxes. Factorial ANOVA with *GI* versus *gi* and accession as main factors yielded significant effects of the *gi* mutations at $P < 0.0001$ for each response (cotyledon unfolding or hypocotyl growth) and light condition (R or FR). In the case of L*er*, only *gi-5* was included in the analysis to maintain the balance of one wild type and one mutant per accession.

at high fluences of continuous FR indicates that HIR is unimpaired in *gi* (Huq et al., 2000; Tseng et al., 2004).

The induction of seed germination by a brief pulse of FR is a typical VLFR mediated by phyA (Botto et al., l996; Shinomura et al., 1996). Based on the observed seedling phenotype under hourly pulses of FR, we investigated whether the VLFR of seed germination was also affected in *gi*. The seeds were incubated for 3 d at 6°C followed by 3 h at 37°C, transferred to 25°C, and exposed to either a brief pulse of long-wavelength FR to establish a very low level of the Pfr form of phytochromes or to a pulse of R to establish the maximum proportion of Pfr that is physiologically possible. The *gi* mutants showed reduced promotion of seed germination by a long-wavelength FR pulse compared to dark controls (Fig. 2). A pulse of R promoted virtually full germination in the wild type and the *gi* mutant alleles. Thus, *gi* mutants are impaired in the VLFR of seed germination. To analyze an additional phyA-mediated response, seedlings of Arabidopsis were grown either in darkness or under hourly pulses or continuous long-wavelength FR and subsequently transferred to continuous white light as described by Luccioni et al. (2002). In the wild type, FR given for several days impaired subsequent accumulation of chlorophyll upon transfer to white light (Barnes et al., 1996). Chlorophyll levels measured after 2 d under white light did not differ between the wild type and the *gi* mutant (data not shown), indicating that this response does not obviously require GI.

GI is involved in the control of circadian rhythms, likely via roles in blue and R input to the clock and within the central oscillator itself (Fowler et al., 1999; Park et al., 1999; Eriksson and Millar, 2003; Locke et al., 2005; Mizoguchi et al., 2005; Martin-Tryon et al., 2007). The circadian clock is known to affect plant sensitivity to R, a phenomenon known as ‘‘gating’’ (Millar and Kay, 1996). The observed phenotype of *gi* seeds in response to a FR pulse could result from improper gating of the light response rather than a defect in light signaling. To investigate this possibility, chilled seeds were transferred to 22°C (darkness) and exposed to a 5-min pulse of FR at different time points of the first two 24-h cycles. The induction of germination of wild-type seeds by FR showed a first maximum peak early during the first subjective night (21 h) and a second peak 20 h later (i.e. 41 h), suggesting that the sensitivity to phyA is gated by the circadian clock (Fig. 3). The apparent period of less than 24 h could result from shortening of the period in darkness or from the overall tendency of reduced germination after extended time in darkness (compare first and second days). The *gi-5* mutant showed reduced induction of germination by pulses of FR and approximately followed the fluctuations of the wild type during the first 24 h after chilling, but *gi-5* germinated poorly and showed no clear rhythm during the second cycle (Fig. 3). A pool of *gi-5* seed characterized by high germination in darkness still showed reduced VLFR of seed germination (difference between FR pulses and darkness) compared to the wild type but with a more defined peak of sensitivity during the second 24-h cycle (Fig. 3). To focus the attention on the rhythmic pattern of sensitivity, we normalized the germination response. We divided the percentages of germination of each genotype by the average for each 24-h cycle to de-trend the output (Levine et al., 2002). Then we set the lowest de-trended value of each genotype to zero and the highest to one. Both genotypes showed a maximum peak at 21 h and a second peak 20 h later (Fig. 3, inset). This indicates that the *gi* mutation does not affect the rhythm of sensitivity to FR pulses and suggests that reduced germination sensitivity in *gi* is not due to defects in the circadian system.

To investigate whether the effect of GI on other physiological processes involves changes in the gating by the clock, we exposed entrained seedlings to pulses of FR given at different time points of the 24-h cycle. To synchronize the rhythms, the seeds were sown at 7 PM local time because imbibition sets the clock to an evening phase (Zhong et al., 1998). The seeds were incubated 61 h in darkness at 5°C and given 11 h R to induce germination and returned to darkness (22°C), i.e. the termination of the R treatment coincided with the hour of seed imbibition to reinforce the evening phase signal (or at least to avoid a contradictory signal). The seedlings remained in darkness during a variable amount of time, received five consecutive hourly FR pulses (3 min), and were returned to darkness (Fig. 4). Two days later, the distance between the cotyledons was measured under magnifying glass instead of the angle between cotyledons because the effects of only five pulses could not be resolved with a protractor. The wild type showed a biphasic fluctuation in sensitivity to FR pulses with maximum peaks at the end of the subjective night and at the end of the subjective day

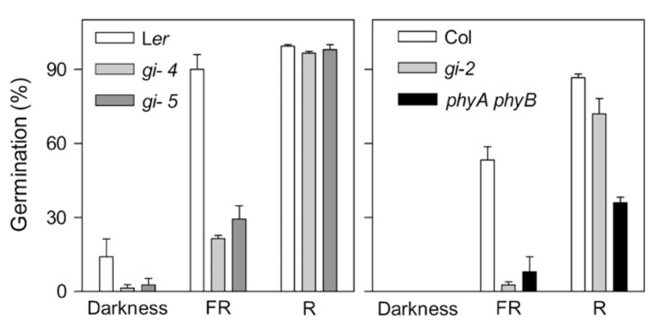

Figure 2. The VLFR of seed germination is reduced in *gi* compared to the wild type. Chilled seeds were exposed to a pulse of either long-wavelength FR or R and returned to darkness for 3 d before counting germinated seeds. Data are means and SE of two (left) or three (right) replicate boxes. Factorial ANOVA yielded significant ($P < 0.0001$) interactions between light condition and genotype in both cases. Bonferroni tests indicate significant differences between *gi-4* and wild type in darkness ($P < 0.05$) and in response to a FR pulse ($P < 0.001$), between *gi-5* and wild type only in response to a FR pulse ($P < 0.001$), and between *gi-2* and wild type in response to a FR ($P < 0.001$) or R ($P < 0.05$) pulse.

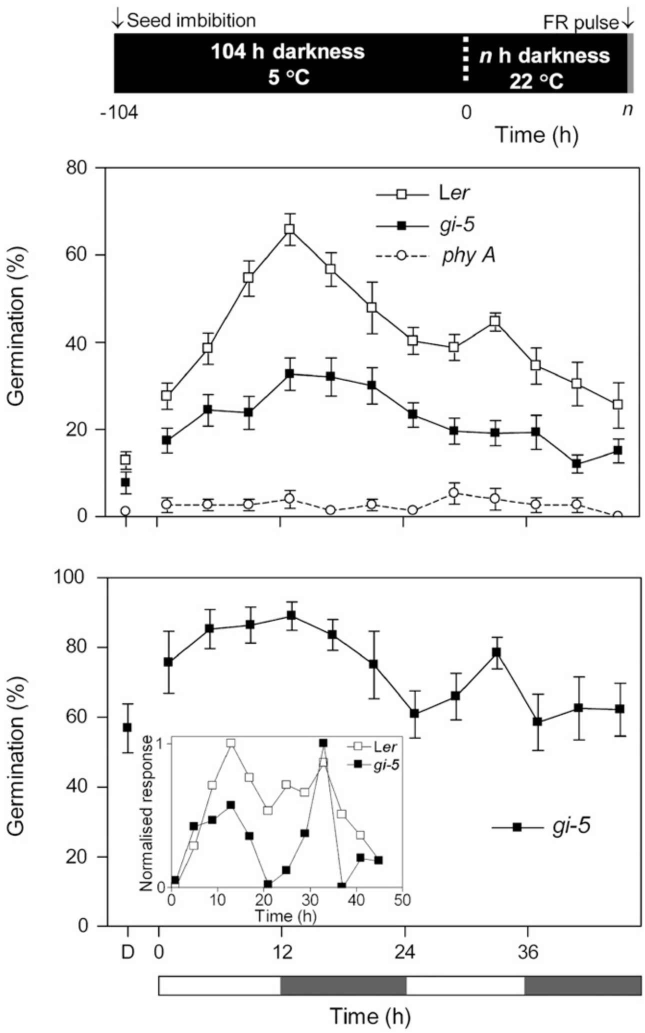

Figure 3. The circadian clock gates VLFR of seed germination but *gi* does not affect the phase of rhythmic sensitivity. The seeds were sown at 7 PM, incubated 104 h in darkness at 5°C, and transferred to 22°C (always in darkness) for the time indicated in abscissas, before a 5-min pulse of FR. The number of germinated seeds was counted 4 d later. White and gray boxes represent subjective day and night, respectively. Data are means and SE of 12 replicate boxes. Factorial ANOVA of the data shown in the top panel indicates that the effect of *GI* versus *gi* is significant at $P < 0.0001$; the effect of time is significant at $P < 0.0001$ and the interaction is significant at $P < 0.04$. Seeds of the wild type or of the *gi* mutant exposed to a pulse or R at time = 0 germinated more than 90%.

(Fig. 4). The *gi* mutant showed a reduced response to pulses of FR but the rhythmic fluctuations paralleled those of the wild type, indicating circadian gating of this VLFR was not affected by *gi*.

We also investigated whether the *gi* mutation affects FR-mediated induction of expression of a chlorophyll *a/b*-binding protein (*CAB*) gene. The induction of *CAB* gene expression by a FR pulse is a VLFR mediated by phyA (Hamazato et al., 1997). We made use of *CAB2*: *LUC* plants, in which expression of firefly luciferase is regulated by the *CAB2* promoter, which have been extensively investigated in connection with circadian rhythms. Wild-type and *gi* plants expressing this transgene were exposed to pulses of FR at different times of the subjective day and night. The wild type showed peaks of response that corresponded to the expected time points based on previous experiments using pulses of R (Millar and Kay, 1996), while the *gi-201* mutant showed the same pattern of temporal fluctuation but with reduced luciferase activity (Fig. 5). Preliminary experiments with the *gi-2* mutant indicate a similar pattern (data not shown). This indicates that GI affects phyA-mediated responses to pulses of FR independently of its effects on clock functions.

The hypocotyl of etiolated seedlings grows against the gravity vector. R or FR perceived by phytochromes decrease the negative gravitropic stimulation and the hypocotyl adopts a randomized position (Poppe et al., 1996; Robson and Smith, 1996; Hangarter, 1997). Hypocotyl angle relative to the vertical position was small in dark-grown wild-type, *phyA*, *g*i, *phyA gi*, and *phyB* seedlings (Fig. 6). Hourly pulses of FR increased the average angle via a phyA-mediated response unaffected by *gi* (Fig. 6). Pulses of R significantly increased hypocotyl angle compared to pulses of FR, except in the *phyB* mutant. This indicates that the difference

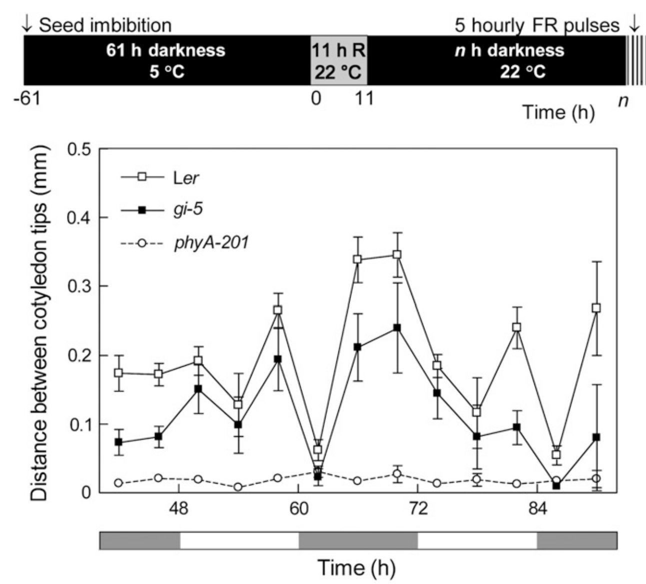

Figure 4. *gi* affects the sensitivity of cotyledon unfolding in response to light, but not circadian gating of this response. The seeds were sown at 7 PM, incubated 61 h in darkness at 5°C, and given 11 h R to induce germination. Seed imbibition synchronizes biological rhythms to the beginning of the night phase (Zhong et al, 1998), and this coincided with the time when the R treatment to induce germination was terminated. Time 0 in abscissas is the beginning of the R treatment. White and gray boxes represent subjective day and night, respectively. The seedlings were exposed to five pulses of FR (3 min) starting at the time indicated in abscissas. The distance between cotyledons was measured 2 d later. Data are means ± SE of six replicate boxes. Factorial ANOVA indicates that the effect of genotype is significant at $P < 0.0001$; the effect of time is significant at $P < 0.0001$ and the interaction is not significant.

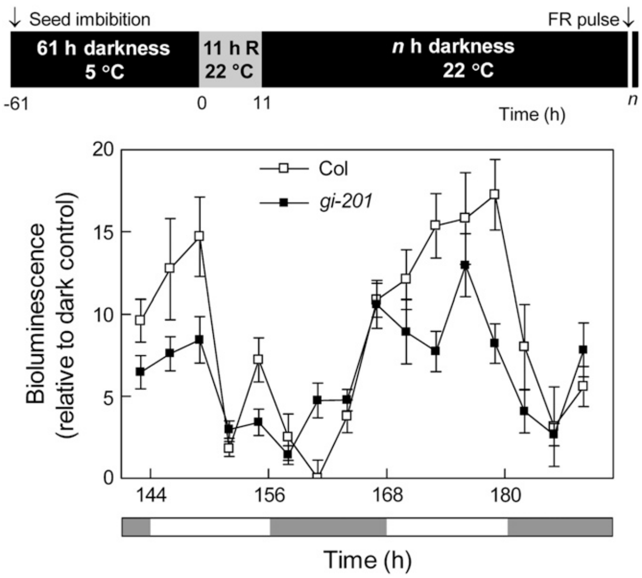

Figure 5. The response of *CAB2*::*LUC* activity to a pulse of FR is affected in the *gi-201* mutant. The seeds were sown at 7 PM, incubated 61 h in darkness at 5°C, and given 11 h R to induce germination. Time 8 h in abscissas is subjective dawn of day 5 after the R treatment to induce germination. White and gray boxes represent subjective day and night, respectively. The seedlings were exposed to a pulse of FR (3 min) at the time indicated in abscissas. Bioluminescence values integrated for 6 h after the pulse are expressed relative to the levels in darkness. Data are means and SE of 50 seedlings. Factorial ANOVA yielded significant effects of time ($P < 0.0001$), genotype ($P < 0.0001$), and interaction ($P < 0.001$).

between R and FR is largely mediated by phyB. Compared to the wild type, the *phyA* mutant had an enhanced response to R consistent with the negative regulation of phyB-mediated responses by phyA, previously observed for other responses (Cerdán et al., 1999). Of note, the *phyA gi* double mutant had an even larger response to R than the *phyA* mutant. This suggests that the reduction of the hypocotyl gravitropic response mediated by phyB is negatively regulated by GI, in contrast to its positive role in other phyB-mediated processes such as inhibition of hypocotyl elongation.

DISCUSSION

The long-hypocotyl phenotype of *gi* observed in seedlings grown under continuous R is not readily apparent under continuous FR (Huq et al., 2000; Tseng et al., 2004). Since the inhibition of hypocotyl elongation by continuous R is mediated largely by phyB (Reed et al., 1993; Quail et al., 1995) with only a minor contribution from phyA (Mazzella et al., 1997), GI would appear as a positive regulator of phyB signaling during seedling de-etiolation without playing a major role in phyA signaling (Huq et al., 2000; Tseng et al., 2004), despite the promotion of *GI* expression by phyA (Tepperman et al., 2001). However, by exploring a different set of photobiological and physiological responses, present experiments extend the action of GI to the regulation of phyA signaling. All the *gi* alleles included in the experiments reported here are hyposensitive for the hypocotyl-growth inhibition and cotyledon-unfolding responses to hourly pulses of FR (Fig. 1), a VLFR mediated by phyA (Yanovsky et al., 1997; Supplemental Fig. S1). This phenotype is rescued by ectopic expression of *GI* (Fig. 1). The *gi* mutants also showed weak induction of seed germination (Fig. 2) and *CAB2*::*LUC* activity (Fig. 5) in response to a pulse of FR, which are typical VLFRs mediated by phyA (Botto et al., l996; Shinomura et al., 1996; Hamazato et al., 1997). However, the blocking of greening (Barnes et al., 1996; Luccioni et al., 2002) and the inhibition of negative gravitropism of the hypocotyl induced by pulses of FR were not affected by the *gi* mutation, indicating that GI selectively affects certain physiological processes under phyA control. Under hourly pulses of FR, only the VLFR pathway is activated. Under continuous FR the HIR dominates the scene, the VLFR accounts for only a weaker contribution retained by mutants that lack the HIR (Yanovsky et al., 2002), and the relative effect of *gi* is maximal at the lowest fluence rates (Supplemental Fig. S2). Since PHYA protein levels are unaffected in *gi* mutants (Huq et al., 2000), GI is a positive regulator of phyA-mediated signaling in the VLFR but not the HIR branch.

In the *phyA* mutant background, *gi* reduced hypocotyl-growth and cotyledon-unfolding responses to R pulses (high Pfr/P in Supplemental Fig. S1), but it enhanced the hypocotyl angle response to R (involving

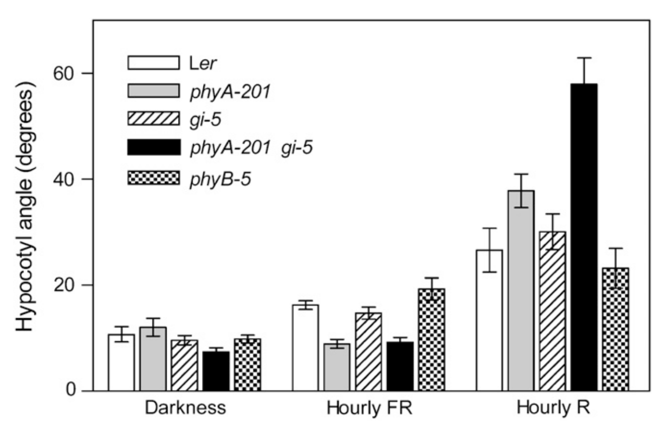

Figure 6. GI affects the hypocotyl gravitropic response in the *phyA* mutant background but not in the presence of phyA. One-day-old seedlings grown on vertical agar were transferred to the indicated light treatments for 3 d before measurements of the angle between the hypocotyl and the vertical axis. Data are means and SE of 12 replicate boxes. Factorial ANOVA (*GI/gi* and *PHYA/phyA* as main factors) was conducted for each light and dark condition (the *phyB* mutant was not included in the analysis). In darkness, the effect of *GI* versus *gi* was significant at $P < 0.05$. Under hourly FR, the effect of *PHYA* versus *phyA* was significant at $P < 0.0001$. Under hourly R, the interaction was significant at $P < 0.04$ because the effect of *gi* versus *GI* was significant only in the *phyA* background.

negative regulation of gravitropism; Poppe et al., 1996; Robson and Smith, 1996; Hangarter, 1997; Fig. 6). The residual morphological effects observed in the *phyA* mutant background are largely mediated by phyB (Cerdán et al., 1999). Therefore, GI would be a positive or negative regulator of phyB signaling, depending on the physiological process under consideration.

Several genes, including *ELF3* (Covington et al., 2001; Hicks et al., 2001; Liu et al., 2001), *ELF4* (Khanna et al., 2003), *TOC1/APRR1* (Más et al., 2003), *APRR5* (Sato et al., 2002; Yamamoto et al., 2003), *APRR7* (Kaczorowski and Quail, 2003; Yamamoto et al., 2003), *CCA1* (Wang and Tobin, 1998), *LHY* (Schaffer et al., 1998), *SRR1* (Staiger et al., 2003), *ZTL* (Somers et al., 2004), and *GI* (Fowler et al., 1999; Park et al., 1999; Huq et al., 2000), affect circadian rhythms and photomorphogenesis. The effect of ELF3 on light responses is largely mediated by its control of rhythmic sensitivity to light (Dowson-Day and Millar, 1999; McWatters et al., 2000; Covington et al., 2001), but whether a similar hierarchy between rhythms and light responses is true for the other aforementioned genes is unknown. To investigate whether GI affected phyA-mediated responses by modifying the gating pattern, the rhythms were entrained at the seed stage, and the *CAB2*::*LUC* seedlings grown under free running conditions (darkness, constant temperature) were exposed to a pulse of FR. The peaks of response occurred during the subjective day as predicted from previous experiments using a pulse of R (Millar and Kay, 1996) both in wild-type and *gi* mutant seedlings (Fig. 5). Seedlings entrained as in the *CAB2*::*LUC* experiments were also exposed to five successive hourly pulses of FR given at different times of the subjective day/night. In this case, the extent of cotyledon unfolding of wild-type and *gi* seedlings showed a peak at the end of the subjective night and a second bout of high responsivity at the end of the subjective day (Fig. 4). There are other cases where circadian rhythms result in two peaks per cycle (Kolar et al., 1998; Strayer et al., 2000), which could result from the action of diverse clock output components. Finally, the induction of seed germination by a pulse of FR showed a maximum peak in the subjective evening, indicating that the VLFR of seed germination is also under the control of the circadian clock (Fig. 3). Since the *CAB* gene-induction, cotyledon-unfolding, and seed-germination responses to pulses of FR are mediated solely by phyA (Botto et al., l996; Shinomura et al., 1996; Hamazato et al., 1997; Yanovsky et al., 1997), present results indicate that a circadian rhythm gates phyA-mediated responses. VLFR can synchronize the clock (Nagy et al., 1993) and the clock gates VLFR (Figs. 3–5). The *gi* mutants showed reduced responses to the pulses of FR, but the temporal pattern of these responses was undistinguishable from that of the wild type (Figs. 3–5). We do not exclude effects of GI on photomorphogenesis mediated by its role in the circadian clock. However, we conclude that GI can positively regulate the VLFR pathway of phyA signaling via pathways not mediated by clock regulation. A comparable scenario has been proposed for the action of GI in the promotion of flowering, which does not appear to be mediated by the regulation of circadian rhythms by GI (Mizoguchi et al., 2005; Martin-Tryon et al., 2007). The differences between wild-type and *gi* seedlings were somewhat larger at the times of the daily cycle with higher sensitivity to FR pulses (Figs. 3 and 4). GI is therefore necessary for the full display of the gating of VLFR by the clock. Since *GI* expression shows a circadian rhythm with a maximum peak in continuous darkness 12 h after the beginning of the subjective day (Fowler et al., 1999) and protein levels follow very closely the levels of transcript (David et al., 2006), GI itself could contribute directly to the gating process enhancing evening responsivity (Figs. 3 and 4). phyA (Tóth et al., 2001), SPA1 (Harmer et al., 2000), and AFR (Harmon and Kay, 2003) also show circadian rhythms of expression and could contribute to the rhythms in sensitivity to FR observed here.

MATERIALS AND METHODS

Plant Material

Plants of Arabidopsis (*Arabidopsis thaliana*) of the accession L*er*, Col, or Ws were used as wild type. Fowler et al. (1999) describe the *gi-2* in Col, *gi-3*, *gi-4*, *gi-5*, and *gi-6* in L*er*, and *gi-11* in Ws. Martin-Tryon et al. (2007) describe the *gi-201* allele in Col. The Arabidopsis Biological Resource Center (ABRC) stocks (Ohio State University) provided seeds of *gi-2* through *gi-6*. Transgenic plants of the *gi-11* Ws background homozygous for a single *GI* transgene under the control of the strong 35-S viral promoter were generated by *Agrobacterium tumefaciens*-mediated transformation. David et al. (2006) have described the three *gi-2* lines expressing the *GI* gene under the control of the 35-S viral promoter (18-42; 20-11 and 12.2). The *phyA-201* (Nagatani et al., 1993) and the *phyB-5* (Reed et al., 1993) mutants were included in some experiments. The *phyA-201 gi-5* double mutant was obtained by selecting plants showing long hypocotyls and fully closed cotyledons under continuous FR and late flowering under long days (16 h) in the F2 generation derived from the cross of the parental lines and in subsequent generations.

Hypocotyl Length and Cotyledon Angle

Fifteen seeds of each genotype were sown on 0.8% (w/v) agar in clear plastic boxes and incubated at 6°C for 5 to 7 d. Chilled seeds were exposed to 1 h R at 22°C to induce homogeneous seed germination and transferred to darkness for 23 h. Then, the seedlings were exposed to different light treatments for 3 d: hourly pulses (3 min) of R (20 μmol m^{-2} s^{-1}, provided by light-emitting diodes), FR (40 μmol m^{-2} s^{-1}, provided by incandescent lamps in combination with a water filter and yellow, orange, red, and blue plastic filters; Paolini 2031), long-wavelength FR (40 μmol m^{-2} s^{-1}, provided by incandescent lamps in combination with an RG9 filter; Schott), R plus FR mixtures (11–30 μmol m^{-2} s^{-1}, provided by incandescent lamps in combination with a water filter and yellow, orange, and red plastic filters with or without a green acetate filter to reduce the proportion of R compared to FR), or continuous FR at different fluence rates. The proportion of Pfr relative to total phytochrome was calculated as described (Casal et al., 1991). Hypocotyl length was measured to the nearest 0.5 mm with a ruler in the 10 longest seedlings of each box. The angle between cotyledons was measured with a protractor using the same 10 seedlings. Seedling data were averaged per box (one replicate) and used for statistics.

In gating experiments the seeds were sown in the plastic boxes at 7 PM, incubated 61 h in darkness at 5°C, and given 11 h R to induce germination. Seed imbibition synchronized biological rhythms to the beginning of the night phase (Zhong et al., 1998), and this coincided with the time when the R treatment to induce germination was terminated. In experiments on cotyledon unfolding, the seedlings were exposed to five consecutive pulses of FR (3 min) given at 1-h intervals starting at different times after the induction of seed germination. The distance between cotyledons was measured under magnifying glass. In preliminary experiments we observed that the distance between the tips of the cotyledons (which is a measure of cotyledon unfolding) increased linearly after the five FR pulses and reached a plateau after 48 h. Thus, in the experiments where the pulses were given at different times, the distance was measured 48 to 60 h after the pulses to record maximum opening of the cotyledons.

Seed Germination

Mature seeds were harvested from plants grown under continuous fluorescent white light (100 μmol m^{-2} s^{-1}) at 25°C and stored in darkness at 5°C for at least 1 month before use. Samples of 25 seeds were sown in the clear plastic boxes, exposed to a long-wavelength FR pulse to transform Pfr of stable phytochromes to Pr, wrapped in black plastic, and incubated for 3 d at 6°C. Chilled seeds were exposed to a 5-min (30–40 μmol m^{-2} s^{-1}) pulse of either R or long-wavelength FR or remained in full darkness (without exposure to green light). Then the seeds were incubated in darkness at 25°C for 4 d before counting germinated seeds. Handling of the seeds was in absolute darkness. In gating experiments, the seeds were incubated in darkness at 5°C for 104 h and transferred to 22°C for a period of variable duration before exposure to a 5-min pulse of FR.

Angle of the Hypocotyl

The seeds were sown on agar along a line close to the middle of the plastic boxes, chilled, given an inductive R pulse, and transferred to darkness as described above. Then the boxes were placed vertically under the light treatments (i.e. the agar was shifted from a position parallel to the soil to the normal). The angle of the hypocotyls with respect to the normal position was recorded by placing the clear boxes on a protractor because the seedlings grow parallel to the surface of the agar under the described conditions.

CAB2::LUC

The seeds were sown at 7 PM on agar plates containing Murashige and Skoog and 3% Suc, and incubated in darkness 61 h at 5°C. The plates were transferred to 22°C, exposed 11 h to R, and then incubated in darkness before exposure to a single FR (3-min) pulse. Bioluminescence was measured as described by Martin-Tryon et al. (2007) before and after the FR pulse and in dark controls that never received a FR pulse. The bioluminescence values during 6 h after the FR pulse are expressed relative to the bioluminescence in darkness.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effects of *gi* on cotyledon unfolding and hypocotyl growth in the *PHYA* and *phyA* backgrounds.

Supplemental Figure S2. The maximum effects of the *gi* mutation on the cotyledon-unfolding or hypocotyl-growth responses are attained at very low irradiances of continuous FR and are not increased by more intense light treatments.

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