PHYTOCHROME KINASE SUBSTRATE4 Modulates Phytochrome-Mediated Control of Hypocotyl Growth Orientation[1][W][OA]

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Gravity and light are major factors shaping plant growth. Light perceived by phytochromes leads to seedling deetiolation, which includes the deviation from vertical hypocotyl growth and promotes hypocotyl phototropism. These light responses enhance survival of young seedlings during their emergence from the soil. The PHYTOCHROME KINASE SUBSTRATE (PKS) family is composed of four members in Arabidopsis (*Arabidopsis thaliana*): PKS1 to PKS4. Here we show that PKS4 is a negative regulator of both phytochrome A- and B-mediated inhibition of hypocotyl growth and promotion of cotyledon unfolding. Most prominently, *pks4* mutants show abnormal phytochrome-modulated hypocotyl growth orientation. In dark-grown seedlings hypocotyls change from the original orientation defined by seed position to the upright orientation defined by gravity and light reduces the magnitude of this shift. In older seedlings with the hypocotyls already oriented by gravity, light promotes the deviation from vertical orientation. Based on the characterization of *pks4* mutants we propose that PKS4 inhibits changes in growth orientation under red or far-red light. Our data suggest that in these light conditions PKS4 acts as an inhibitor of asymmetric growth. This hypothesis is supported by the phenotype of PKS4 overexpressers. Together with previous findings, these results indicate that the PKS family plays important functions during light-regulated tropic growth responses.

Plants have evolved sensitive mechanisms to detect and respond to their environment. Among the numerous environmental stimuli plants are particularly sensitive to light (Chen et al., 2004) and gravity (Esmon et al., 2005). While gravity is constant in intensity and is unidirectional, light is highly variable in direction, intensity, spectral composition, and duration (photoperiod). Our understanding of the molecular basis of light and gravity perception has considerably advanced over the past decades, however, we still know little about the mechanisms enabling plants to integrate all this information.

Light is critical for plants because they depend on it as a source of energy. To adjust growth and development to the environmental conditions plants sense UV-B, blue, red, and far-red signals (Chen et al., 2004; Ulm and Nagy, 2005; Jiao et al., 2007). The phytochromes, which sense red and far-red light, are important for several developmental transitions and for adaptive responses such as the shade-avoidance syndrome or the modulation of tropic growth responses (Chen et al., 2004; Iino, 2006; Mathews, 2006). In particular, during deetiolation the growth of hypocotyls is inhibited and the angle between the cotyledons is increased via different photoresponses mediated by the phytochromes. The high-irradiance response (HIR) requires prolonged excitation of phytochrome A (phyA) with far-red light (Nagatani et al., 1993; Whitelam et al., 1993; Quail et al., 1995; Shinomura et al., 2000). The very-low-fluence response (VLFR) is a photobiologically discrete phase of phyA-mediated hypocotyl response, which in contrast to HIR saturates with infrequent excitation (Casal et al., 2000). The hypocotyl growth response to pulses of red light that can be back reverted by subsequent far-red light pulses is the low-fluence response (LFR) mediated by phytochrome B (phyB; Mazzella et al., 1997).

The perception of gravity typically directs root growth downwards while shoots grow upwards. These tropic growth responses maximize the acquisition of essential resources such as water, minerals, and light

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(Blancaflor and Masson, 2003; Esmon et al., 2005). There are, however, numerous exceptions to this rule such as strawberry stems for instance. Digby and Firn (1995) proposed the general concept of gravitational set-point angle (GSA) to define the position at which a nonvertically oriented organ is in equilibrium and shows no gravity-induced differential growth. Genetic analyses in Arabidopsis (*Arabidopsis thaliana*) have identified a number of genes that are essential for the response to gravity (Blancaflor and Masson, 2003; Esmon et al., 2005). Light control of gravitropism has been reported in numerous species and for diverse plant organs (Hangarter, 1997; Digby and Firn, 2002). Light-induced opening of the apical hook in etiolated seedlings is a striking example (MacDonald et al., 1983). The most apical part of the hypocotyl goes from positive gravitropism in darkness (to maintain the apical hook) to negative gravitropism as the hook opens in response to light (MacDonald et al., 1983).

Light perceived by the phytochromes and the blue-light receptor cryptochromes induces the deviation from hypocotyl vertical growth (Poppe et al., 1996; Robson and Smith, 1996; Hangarter, 1997; Lariguet and Fankhauser, 2004; Ohgishi et al., 2004; Whippo and Hangarter, 2004; Iino, 2006; Nagashima et al., 2008). This effect of light on hypocotyl growth orientation appears to maximize the phototropic potential of the hypocotyl (Lariguet and Fankhauser, 2004) and thus confer a competitive advantage to young seedlings grown in patchy, low-light environments (Allen et al., 2006). The mechanisms underlying phytochrome control of hypocotyl orientation remain poorly understood and although it is generally presented as phytochrome-mediated inhibition of hypocotyl gravitropism, this relationship between hypocotyl orientation and gravitropism has not been formally demonstrated in Arabidopsis (Hangarter, 1997; Digby and Firn, 2002). Interestingly, in rice (*Oryza sativa*) red light does not lead to randomization of hypocotyl growth orientation; red light rather enhances hypocotyl gravitropism (Yoshihara and Iino, 2005). Alternative explanations include effects of light on circumnutation, autostraightening, or a combination of different effects (Poppe et al., 1996; Robson and Smith, 1996; Hangarter, 1997; Lariguet and Fankhauser, 2004; Ohgishi et al., 2004; Whippo and Hangarter, 2004; Iino, 2006). Recently, the *GIL1* gene coding for a plant-specific protein of unknown function has been identified as a specific component of phytochrome-mediated control of hypocotyl growth-orientation (Allen et al., 2006). Two related bHLH class transcription factors that affect numerous phyA-mediated responses and the ABC-type auxin transporter PGP19 have also been implicated in the control of hypocotyl growth orientation (Fairchild et al., 2000; Oh et al., 2004; Nagashima et al., 2008).

Although initially identified for their function in phytochrome signaling (Fankhauser et al., 1999; Lariguet et al., 2003; Khanna et al., 2006), *pks* (phytochrome kinase substrate) mutants display severe defects in hypocotyl phototropism (Lariguet et al., 2006) as well as root photo- and gravitropism (Boccalandro et al., 2008). Moreover, PKS1 interacts with phototropin1 (phot1), a receptor of blue-light gradients that triggers phototropism, and an essential phototropism signal transduction element NONPHOTOTROPIC HYPOCOTYL3 (NPH3; Lariguet et al., 2006). Given this scenario, we decided to investigate whether PKS proteins also affect phytochrome-regulated growth orientation. Our results show that PKS4 controls phytochrome-mediated hypocotyl growth orientation. Moreover the phytochromes have distinct effects on this response depending on the developmental stage of the seedling at which it is exposed to light. When given immediately after germination, red light inhibits negative hypocotyl gravitropism. In contrast, when dark-grown seedlings with hypocotyls already displaying negative gravitropism are exposed to red light, this leads to deviation from vertical growth without affecting the gravi-reorientation potential of the hypocotyl.

RESULTS

The name PKS4 (At5g04190) is based on homology with the other members of the *PKS* gene family, but the ability to interact with phytochrome had not been tested previously (Lariguet et al., 2006). Here we show that, similarly to PKS1 and PKS2, PKS4 interacts with phyA and phyB in yeast two-hybrid analysis and in vitro pull-down assays (Supplemental Fig. S1).

Regulation of *PKS4* Transcript Abundance in Seedlings

To investigate whether the expression of *PKS4* is regulated by light during deetiolation, 4-d-old seedlings were transferred from darkness to different light conditions (Supplemental Fig. S2). Red and blue light led to a rapid and transient decrease in transcript abundance whereas far-red light led to a slower and progressive decline of *PKS4* mRNA levels (Supplemental Fig. S2). The reduced responses of the *PKS4* mRNA level in the *phyA* mutant demonstrated a role for phyA in the regulation of *PKS4* transcript abundance by light (Supplemental Fig. S2). *PKS1* and *PKS2* have distinct expression patterns in young seedlings, with *PKS1* being expressed in the elongation zone of both hypocotyls and roots, and *PKS2* being primarily expressed in the cotyledons (Lariguet et al., 2003). It was thus of interest to determine the expression pattern of *PKS4*. To do so we generated transgenic lines expressing the GUS reporter gene under the control of the *PKS4* promoter. Both in etiolated seedlings and dark-grown seedlings transferred for several hours to light *PKS4* was mainly expressed in the hypocotyl elongation zone (Fig. 1). This spatial expression pattern is similar to the one reported for *PKS1*, except that *PKS4* is not expressed in the root elongation zone. Moreover, light-dependent down-regulation of *PKS4* was not observed in *PKS4*:*GUS* lines (data not shown),

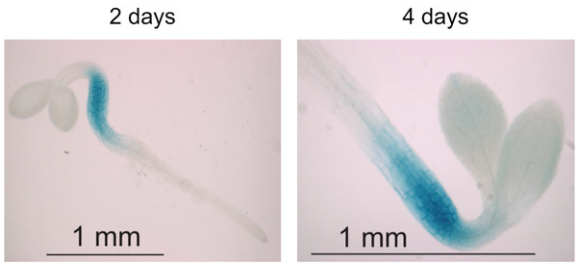

Figure 1. *PKS4* is expressed in the hypocotyl elongation zone. GUS-staining of 2-d-old or 4-d-old dark-grown seedlings. The blue staining indicates the expression of the reporter protein.

which is presumably due to the strong stability of the GUS reporter.

PKS4 Regulates VLFR, LFR, and HIR of Hypocotyl Growth

PKS1 and PKS2 negatively regulate one specific branch of phyA signaling, the VLFR (Lariguet et al., 2003). Seedlings with all possible mutant combinations between *pks1*, *pks2*, and null alleles of *pks4* (Lariguet et al., 2006) were subjected to specific light regimes that selectively induced the phyA-mediated VLFR, the phyB-mediated LFR, or the phyA-mediated HIR (Lariguet et al., 2003). These experiments confirmed the enhanced VLFR in *pks1* and *pks2* mutants and showed that *pks4* mutants have a similar phenotype (Fig. 2). However, in contrast to PKS1 and PKS2, PKS4 also regulates LFR and HIR of hypocotyl elongation and cotyledon opening (Fig. 2).

The analysis of double and triple mutants indicated a complex set of genetic interactions between the three members of the *PKS* gene family. We confirmed the reversion of the *pks1* and *pks2* mutant phenotypes in the *pks1pks2* double mutant (Fig. 2; Lariguet et al., 2003). Generally speaking we observed no additive effects of the different *pks* phenotypes and the multiple mutants including *pks4* essentially looked like the *pks4* single mutant (Fig. 2). While the *pks4* mutants showed enhanced VLFR and LFR, *PKS4* overexpressing seedlings showed reduced VLFR and LFR (Fig. 3). Double mutants between *pks4* and *phyA* or *phyB* showed that the photoreceptor mutations were epistatic to the *pks4* mutation under conditions where phyA or phyB mediates the light responses (Fig. 3). We conclude that PKS4 regulates phyA- and phyB-mediated deetiolation.

PKS4 Controls Light-Mediated Deviation from Vertical Hypocotyl Growth

The previously identified function of the *PKS* genes during phototropism prompted us to analyze the possible function of these genes during another light-controlled tropic response: phytochrome-mediated deviation from vertical hypocotyl growth (Poppe et al., 1996; Robson and Smith, 1996; Whippo and Hangarter, 2004; Lariguet et al., 2006; Nagashima et al., 2008). The negative gravitropic response displayed in dark-grown seedlings is normal in the *pks* mutants (Lariguet et al., 2006; see also Fig. 7). When grown in monochromatic red or far-red light to analyze the phytochrome effect on hypocotyl orientation, the *pks4* mutants displayed a reduced light-induced deviation from vertical growth, the *pks2* mutant behaved as the wild type and the *pks1* mutant showed a slightly reduced response in red light (Fig. 4). We thus concentrated our analysis on PKS4, which played the strongest role for this light response. To show the effect of light on hypocotyl growth orientation we present

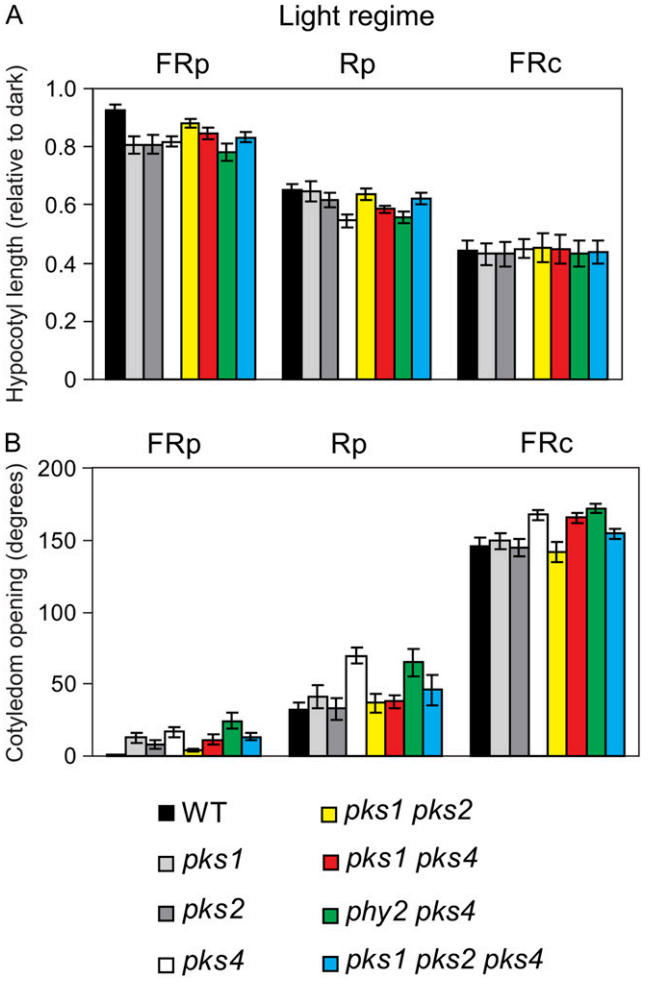

Figure 2. PKS4 is involved in phytochrome-mediated deetiolation responses. One-day-old seedlings of wild type (Col) and of the *pks1*, *pks2*, *pks4*, *pks1pks2*, *pks1pks4*, *pks2pks4*, and *pks1pks2pks4* mutants were exposed to hourly pulses of far-red (FRp; 3 min, 40 μmol m^{-2} s^{-1}), hourly pulses of red light (Rp; 3 min, 40 μmol m^{-2} s^{-1}), or continuous far-red light (FRc; 2.5 μmol m^{-2} s^{-1}) for 3 d. Data are mean of 15 replicate boxes (10 seedlings per box) ± SE. A, Measurements of hypocotyl length relative to dark controls. B, Measurements of cotyledon opening.

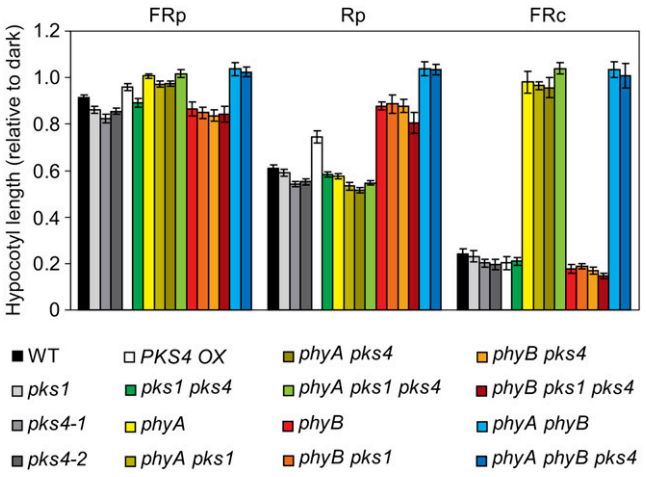

Figure 3. PKS4 acts in phyA and phyB signaling. One-day-old seedlings of wild-type Col, of the *pks1*, *pks4*, and *pks1pks4* mutants and their combinations with *phyA* and/or *phyB* mutants, and of the PKS4 OX were exposed to hourly pulses of far-red (FRp; 3 min, 40 μmol m^{-2} s^{-1}), hourly pulses of red light (Rp; 3 min, 40 μmol m^{-2} s^{-1}), or continuous far-red light (FRc; 2.5 μmol m^{-2} s^{-1}) for 3 d before measurements of hypocotyl length, which is presented relative to dark controls. Data are means ± SE of 15 replicate boxes (10 seedlings per box).

the average angle of the hypocotyl and the 95% confidence limits to describe the range of positions adopted by this organ under conditions that cause different degrees of randomization.

Light Control of Hypocotyl Growth Orientation Depends on the Developmental Context

Negative gravitropism of dark-grown hypocotyls is not simply a passive default state. To grow against the gravity vector, the hypocotyl must reorient, unless if by chance the seed position already aligns the hypocotyl growth axis with the gravity vector. In Figure 5A, the original orientation of the radicle and of the hypocotyl of a dark-grown seedling is essentially horizontal. After emergence, the root rapidly shifted the direction of growth following the gravity vector. The hypocotyl also changed the direction of growth very close to its point of emergence from the seed, adopting a near vertical position. If the seedling is exposed to light at early stages of development, the growth of the hypocotyl adopts a direction largely influenced by seed position (Fig. 5B). In Figure 5B the radicle emerged toward the right and the hypocotyl grew toward the opposite side. The effect of light can thus be seen as an inhibition of the hypocotyl asymmetric growth response required for gravitropism. To test this hypothesis we positioned seeds with the embryo (longest axis) oriented horizontally and the micropyl either toward the right- or the left-hand side. The micropyl is the place of radicle emergence and the shoot apical meristem is located at the opposite extreme of the longitudinal axis of the seed. We then recorded the final growth orientation of those two groups of seedlings separately after 4 d growth in continuous red light (Fig. 5C). The final growth orientation was largely influenced by the initial seed position: if the micropyl was toward the right, the hypocotyl grew toward the left and vice versa (Fig. 5C). Statistical analysis (contingency test) indicates that hypocotyl orientation is very significantly affected by seed position ($P < 2.77 \times 10^{-11}$).

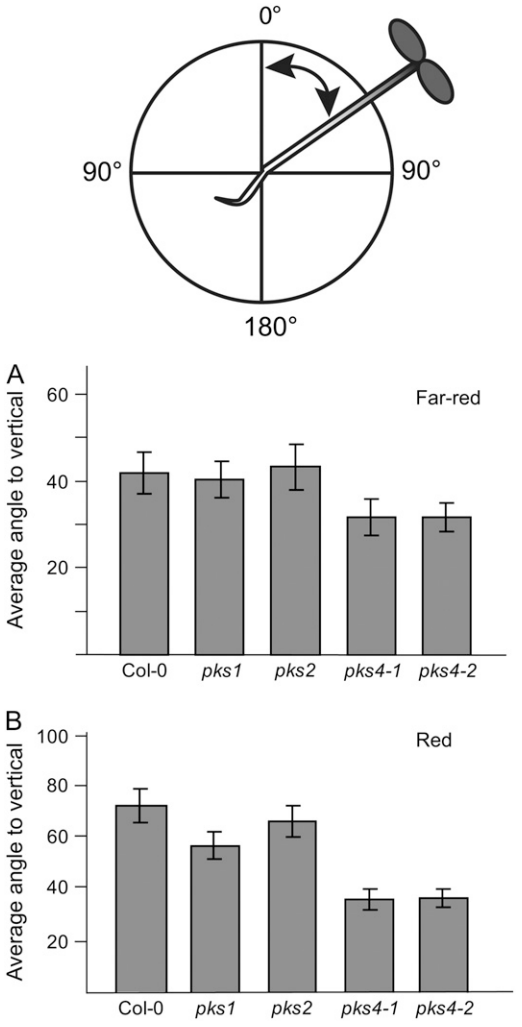

Figure 4. Among the *PKS* genes, *PKS4* has the strongest effect on deviation from vertical growth in red and far-red light. Schematic representation of the method used to measure hypocotyl growth orientation. Note that we consider the same angle whether the seedling bends toward the left or the right side. Thus a totally random distribution should give an average angle of 90°. A, Hypocotyl growth orientation in continuous far-red light (3 μmol m^{-2} s^{-1}). Seedlings were kept for 24 h in the dark prior to 4 d of light treatment. B, Hypocotyl growth orientation in continuous red light (15 μmol m^{-2} s^{-1}). Seedlings were kept for 24 h in the dark prior to 4 d of light treatment; 0° represents vertical growth. Data are mean with 95% confidence intervals indicated; $n > 150$.

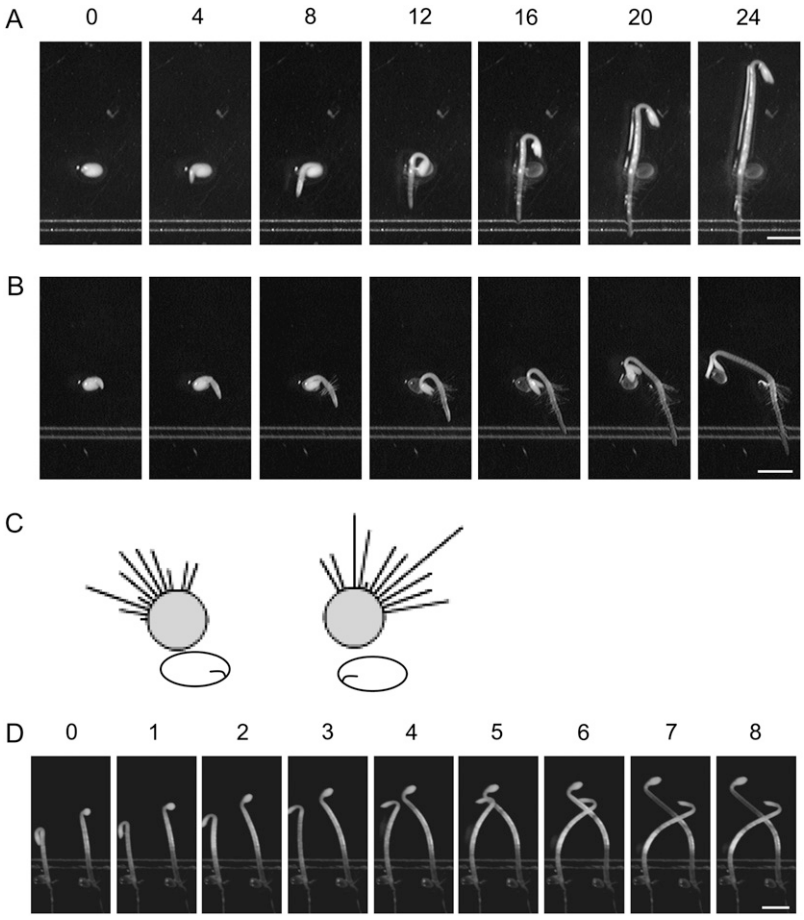

Figure 5. Developmental control of light-mediated modulation of hypocotyl growth orientation. A, Negative gravitropism of the hypocotyls in a dark-grown seedling. Time 0 represents the time of radicle emergence. Note that it emerges toward the left indicating that the embryo axis is positioned horizontally. Pictures are taken every 4 h. Note that the growth axis changes to orient hypocotyl growth against the gravity vector. The scale bar represents 1 mm. B, Seedling development in monochromatic red light (15 μmol m^{-2} s^{-1}). Time 0 represents the time of radicle emergence, pictures are taken every 4 h. Note that the embryo axis is positioned horizontally with the radicle emerging toward the right. Note that the growth axis does not change much in red light. The scale bar represents 1 mm. C, Hypocotyl growth axis of positioned seedlings grown in monochromatic red light (15 μmol m^{-2} s^{-1}). The initial position of the seeds is schematically represented and the final growth axis presented as circular histograms; $n > 125$. Note that the seed position has a big influence on the final hypocotyl growth axis. D, Change in hypocotyl growth direction in etiolated seedlings transferred into red light (15 μmol m^{-2} s^{-1}). Time 0 represents the seedlings after 48 h growth in darkness. Seedlings are imaged every hour once transferred into red light. The scale bar represents 1 mm.

When light was applied after a period of growth in darkness that allowed the hypocotyl to initiate negative gravitropism it induced bending of the hypocotyl at the level of the elongation zone leading to deviation from vertical growth (Fig. 5D). Thus, during early stages of hypocotyl elongation in darkness, gravity perception promoted asymmetric growth and vertically oriented the hypocotyl (Fig. 5A). At later stages light promoted asymmetric growth to cause deviation from vertical growth of a hypocotyl that was growing against the gravity vector prior to the light treatment (Fig. 5D).

The Effects of PKS4 on Hypocotyl Growth Orientation Depend on the Developmental Context

Because the processes involved in light-mediated randomization of hypocotyl growth depend on the developmental context, we investigated the role of PKS4 in hypocotyl growth orientation in seedlings grown for various periods of time in darkness prior to the light treatment. To specifically address phytochrome control of this response, seedlings were transferred to red or far-red light, and growth orientation was recorded after a total of 5 d (Fig. 6). In accordance with the observations described above (Fig. 4), *pks4* seedlings exposed to light shortly after stratification showed reduced deviation from vertical growth compared to the wild type (Fig. 6). Conversely, when the seedlings were grown for 48 h in darkness prior to illumination the *pks4* mutants displayed a larger deviation from vertical growth than the wild type (Fig. 6). The deviation from vertical-growth orientation declined after prolonged periods of growth in darkness presumably because once the hypocotyls were almost fully expanded a change in growth direction could no longer occur (Fig. 6). These experiments suggested apparently opposite roles for PKS4 depending on the time of growth in darkness prior to illumination (Fig. 6). However, in both cases, PKS4 can be viewed as a negative regulator of asymmetric growth (see Fig. 5).

This effect of PKS4 on light-mediated deviation from vertical growth was tested at different fluence rates and both with seedlings that were grown in the light from the time of germination and seedlings that were pretreated with a prolonged growth period in the dark (48 h). Almost irrespective of the fluence rate, *pks4* mutants displayed a greater randomization of hypocotyl orientation when the light was applied after 2-d growth in the dark and a more vertical orientation when they were grown in the light from the time of germination (Fig. 7).

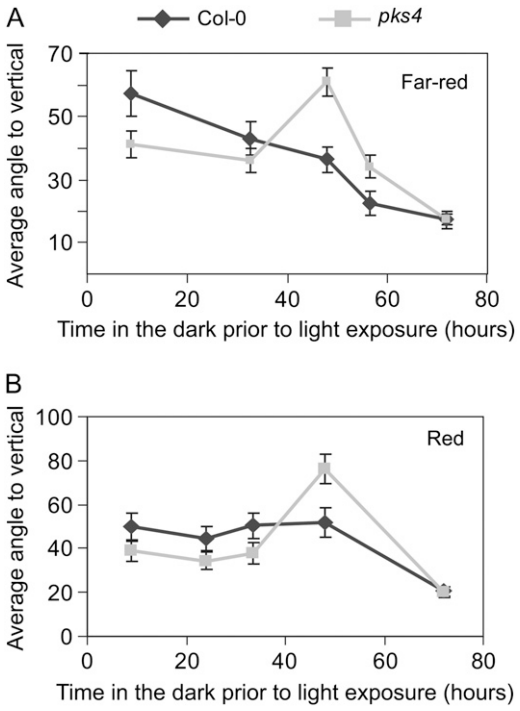

Figure 6. The effect of *PKS4* on hypocotyl growth orientation depends on the time of growth in the dark prior to the light treatment. A, Hypocotyl growth orientation in continuous far-red light (3 μmol m^{-2} s^{-1}). The time of growth in the dark prior to the light treatment is indicated. The growth orientation is determined after 96 h. B, Hypocotyl growth orientation in continuous red light (15 μmol m^{-2} s^{-1}). Experiment performed as in A; 0° represents vertical growth. Data are mean with 95% confidence intervals indicated; $n > 150$.

PKS4 Acts in Phytochrome Signaling during Light-Controlled Hypocotyl Orientation

In far-red light, where phyA is the only photoreceptor significantly contributing to the deetiolation response, the epistatic relationship between *pks4* and *phyA* is easy to interpret. *phyA* was epistatic over *pks4* under all conditions clearly positioning PKS4 in the phyA signaling branch (Fig. 8). In red light, multiple phytochromes participate to the light-induced randomization of hypocotyl orientation (Poppe et al., 1996). Interestingly *pks4* was epistatic over *phyB* whereas *phyA* was epistatic over *pks4*. The comparison between the *phyAphyB* and *phyAphyBpks4* mutants suggests that other phytochromes may participate to the light regulation of hypocotyl growth orientation (Fig. 8).

Overexpression of PKS4 Leads to Constitutive Randomization of Hypocotyl Growth Orientation

Our results based on the phenotype of *pks4* loss-of-function alleles suggested that PKS4 may be necessary to inhibit asymmetric growth during phytochrome modulation of growth orientation. It was thus of interest to determine the growth orientation phenotype of *PKS4* overexpressing seedlings. In agreement with our hypothesis such seedlings displayed an exaggerated randomization of growth orientation in seedlings continuously grown in red and far-red light (Fig. 9, B and C). Interestingly overexpression of *PKS4* led to inhibition of negative gravitropism in etiolated hypocotyls (Fig. 9A).

Light Does Not Reduce the Gravi-Reorientation of Etiolated Hypocotyls

Although generally presented as inhibition of gravitropism, the actual mechanism leading to phytochrome-

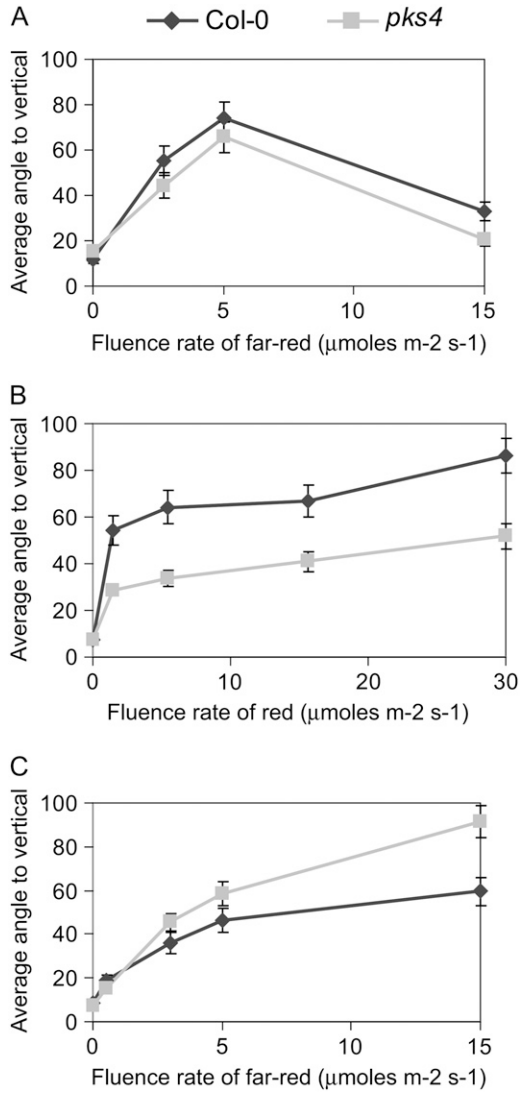

Figure 7. Fluence-rate response curve for hypocotyls growth orientation. A, Hypocotyl growth orientation in continuous far-red light. Seedlings were kept for 24 h in the dark prior to the light treatment. B, Hypocotyl growth orientation in continuous red light. Seedlings were kept for 24 h in the dark prior to the light treatment. C, Hypocotyl growth orientation in continuous far-red light. Seedlings were kept for 48 h in the dark prior to the light treatment; 0° represents vertical growth. Data are mean with 95% confidence intervals indicated; $n > 150$.

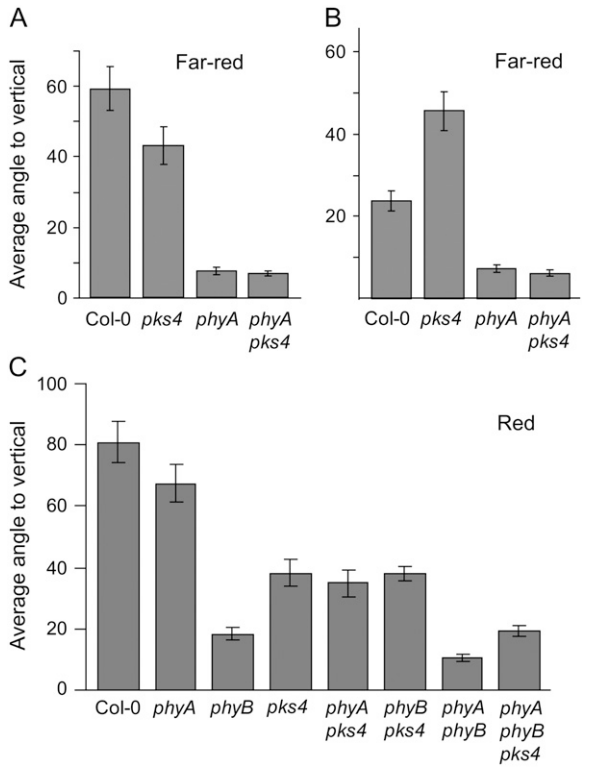

Figure 8. Epistatic relationship between *pks4* and *phy* during the regulation of hypocotyl growth orientation. A, Hypocotyl growth orientation in 3 μmol m^{-2} s^{-1} continuous far-red light. The seedlings were kept in the dark for 24 h prior to the light treatment. B, Hypocotyl growth orientation in 3 μmol m^{-2} s^{-1} continuous far-red light. The seedlings were kept in the dark for 48 h prior to the light treatment. C, Hypocotyl growth orientation in 15 μmol m^{-2} s^{-1} continuous red light. The seedlings were kept in the dark for 24 h prior to the light treatment; 0° represents vertical growth. Data are mean with 95% confidence intervals indicated; $n > 150$.

mediated deviation from vertical hypocotyl growth remains unclear. We investigated whether in 2-d-old etiolated seedlings the light-induced deviation from vertical hypocotyl growth is due to an inhibition of gravitropism. Two-day-old dark-grown seedlings were rotated by 90° and growth reorientation followed by time-lapse imaging over the following 18 h either in darkness or in the presence of red light (Fig. 10). The hypocotyls similarly ($P > 0.05$) reoriented their growth in response to the gravitropic stimulation both in darkness and in red light. However, several light-grown seedlings changed their growth direction after the initial gravi-reorientation and the response was significantly more variable in red-light-grown seedlings than in darkness ($P < 0.05$; Fig. 10, A and B). This variability of the response in red light is presumably related to the one observed in vertically grown seedlings exposed to red light (Fig. 5D). Interestingly, *pks4* seedlings gravistimulated in red light had an early response similar to the wild type but at later time points (after 5 h) there was a greatly enhanced response ($P < 0.005$; Fig. 10B). Conversely, in *pks4* seedlings gravistimulated in the dark, the early gravitropic response was somewhat delayed but reached the same amplitude than in the wild type at later time points. In summary, in hypocotyls displaying negative gravitropism red light caused the deviation of hypocotyl growth direction but it did not reduce the response to gravity stimulation.

DISCUSSION

PKS1 was identified as a protein interacting with and being phosphorylated by phytochrome A in vitro

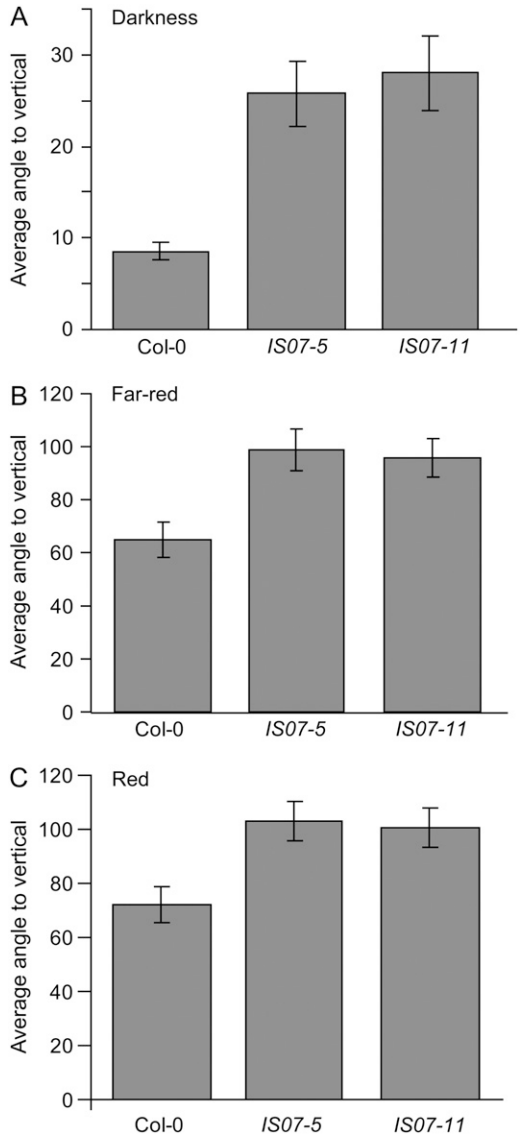

Figure 9. Overexpression of *PKS4* leads to a deviation from vertical growth even in darkness. A, Average hypocotyl growth orientation of 4-d-old dark grown seedlings. B, Hypocotyl growth orientation in 3 μmol m^{-2} s^{-1} continuous far-red light. The seedlings were kept in the dark for 24 h prior to the light treatment. C, Hypocotyl growth orientation in 15 μmol m^{-2} s^{-1} continuous red light. The seedlings were kept in the dark for 24 h prior to the light treatment; 0° represents vertical growth. Data are mean with 95% confidence intervals indicated; $n > 150$.

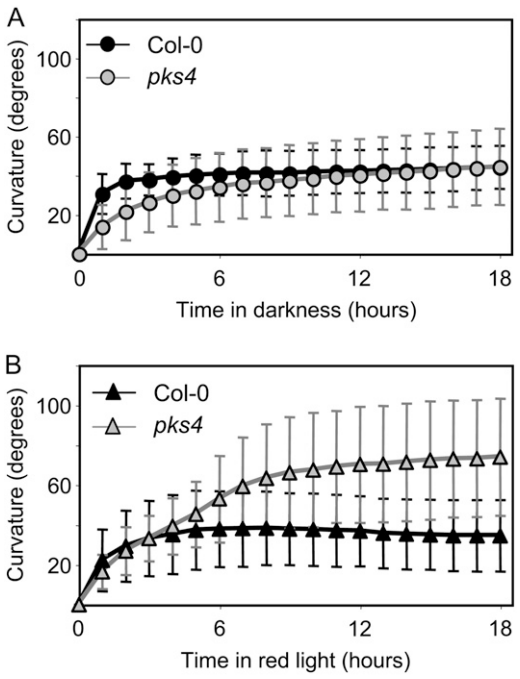

Figure 10. Effect of red light on hypocotyl gravitropism. Changes in hypocotyl growth axis after a 90° rotation of 2-d-old etiolated seedlings either kept in darkness or transferred into monochromatic red light (15 μmol m^{-2} s^{-1}) upon gravistimulation. A, Wild type and *pks4* in darkness. B, Wild type and *pks4* in red light. The angle relative to horizontal (0) is represented as a function of time in hours. Data are mean ± SD; n = 18–20.

(Fankhauser et al., 1999). Physiological studies have shown that PKS1 and PKS2 are required for normal phytochrome-mediated responses (Fankhauser et al., 1999; Lariguet et al., 2003; Khanna et al., 2006). Two additional PKS1-related proteins, PKS3 (At1g18810) and PKS4 (At5g04190), have been identified in Arabidopsis, and PKS-like genes are present in all angiosperms investigated so far (Lariguet et al., 2006). Present data show that among the tested *PKS* genes (*pks3* insertion mutants are not available yet), *PKS4* has the strongest effect on phytochrome-mediated inhibition of hypocotyl elongation and cotyledon opening (Figs. 2 and 3). The expression of *PKS4*:GUS in the hypocotyl elongation zone (Fig. 1) correlates with these physiological responses. Similarly to *pks1* and *pks2* mutants, *pks4* mutants show exaggerated phyA-mediated VLFR (Fig. 2). In addition, and in contrast to PKS1 and PKS2 (Lariguet et al., 2003), PKS4 also negatively regulates the phyA-mediated HIR as well as the phyB-mediated LFR (Figs. 2 and 3).

The phenotypes of *pks4*, *pks1*, and *pks2* are not additive (Fig. 2). A possible explanation for this finding is that for these light responses the PKS proteins work as complexes and elimination of a single member would disrupt their function. Indirect support for this hypothesis comes from a previous study showing that PKS1 and PKS2 interact in vitro (Lariguet et al., 2003). Similarly to PKS1 and PKS2, PKS4 can interact with phyA and phyB in the yeast two-hybrid assay and in vitro (Supplemental Fig. S1). The significance of these interactions remains unclear because we have not been able to verify them in vivo so far. The abundance of *PKS1* and *PKS4* mRNA is light regulated in a phytochrome-dependent manner (Lariguet et al., 2003, 2006; Supplemental Fig. S2). *PKS1* and *PKS4* are the most divergent members of the *PKS* family and it is worth pointing out that their mRNA abundance is regulated almost symmetrically with *PKS1* being rapidly induced by all light conditions while *PKS4* is rapidly down-regulated in response to red and blue but not far-red light (Supplemental Fig. S2). On the contrary, the spatial pattern of gene expression for *PKS1* and *PKS4* in the hypocotyl is highly similar (Fig. 1; Lariguet et al., 2003). Because several phytochrome signaling components show phytochrome-mediated control of expression, the rapid phytochrome-mediated regulation prompted Khanna and coworkers to investigate the function of PKS1 in phytochrome signaling and their work confirmed the importance of PKS1 during deetiolation (Khanna et al., 2006).

In addition to a role in the control of hypocotyl elongation and cotyledon opening we show that PKS4 also regulates phytochrome-mediated control of hypocotyl orientation (Figs. 4–10). Interestingly, PKS4 is most strongly expressed in the hypocotyl elongation zone (Fig. 1), which grows asymmetrically to enable changes in growth orientation. In etiolated seedlings, negative gravitropism of the hypocotyls requires asymmetric growth (Fig. 5A) and gravisensing, given that *arg1* mutant hypocotyls show random growth orientation in darkness (Lariguet et al., 2006). When seedlings are grown in the light from germination, the asymmetric growth that is required to align the hypocotyl growth axis with the gravity vector is inhibited (Fig. 5, B and C). This leads to a deviation from vertical hypocotyl growth orientation, which largely reflects the initial position of the seed (with respect to the gravity vector; Fig. 5C).

When light is applied to dark-grown seedlings that have already initiated negative gravitropism it also leads to deviation from the vertical growth orientation (Fig. 5D). However, the underlying mechanism is different because rather than simply interrupting the gravitropic response and causing the newly emerged hypocotyl to follow the orientation dictated by seed position, light causes the reorientation of distant portions of the hypocotyl that had been previously gravity oriented (Fig. 5D). This change in hypocotyl orientation cannot be fully accounted for by red-light inhibition of the gravitropic response. Actually, when 2-d-old etiolated seedlings were rotated by 90° and reorientation of the hypocotyl monitored either in darkness or light, red light did not inhibit gravireorientation (Fig. 10). Interestingly in rice coleoptiles red light does not inhibit the gravitropic response either (Yoshihara and Iino, 2005). However, as observed for seedlings kept in the vertical position red light leads to a more variable growth orientation (Figs.

5D and 10B), indicating that the response to gravity and red-light-induced change in growth orientation are at least partially different and apparently additive phenomena. Interestingly, when etiolated *pks4* hypocotyls are gravistimulated in red light the initial phase of gravi-reorientation is not significantly different from the wild type (Fig. 10). However, at later time points the response was strongly enhanced and more variable (Fig. 10, B and D) as observed in end-time measurements of 2-d-old etiolated seedlings exposed to light (Fig. 7, B and C). This aspect of phytochrome-mediated reorientation of hypocotyls could involve the control of circumnutation or autostraightening as these processes that influence growth orientation are both modulated by light (Iino, 2006).

The developmental control of light-mediated hypocotyl growth orientation allows us to propose a mode of action for PKS4. Our data suggest that PKS4 is an inhibitor of asymmetric growth in red- or far-red-grown hypocotyls. When seedlings are subjected to light during the phase of asymmetric growth in the hypocotyl elongation zone, removing an inhibitor of asymmetric growth leads to straighter hypocotyls (Fig. 4). In contrast, if light is applied when hypocotyl growth orientation is already aligned with the gravity vector, removing PKS4 (a negative regulator of asymmetric growth) leads to a greater randomization of hypocotyl orientation (Fig. 6). This hypothesis is also consistent with the gravi-reorientation experiments where the *pks4* mutant shows greater reorientation in red light (Fig. 10). Moreover the phenotype of the *PKS4* overexpressing seedlings, which show enhanced randomization of hypocotyl orientation is also consistent with this idea (Fig. 9). Our data from the *pks4* mutants show that if any, PKS4 has a reduced role in the dark, suggesting that in the wild-type PKS4 must somehow be activated by phytochrome (directly or indirectly) to control hypocotyl growth orientation. However, hypocotyls of *PKS4* overexpressing seedlings already show a deviation from vertical growth in darkness indicating that elevated levels or ectopic expression of *PKS4* leads to light-independent negative regulation of asymmetric growth (Fig. 9).

A simple interpretation of the *pks4* loss-of-function mutant phenotype during the control of hypocotyl elongation and cotyledon opening indicates that PKS4 is a negative regulator of phytochrome signaling (Figs. 2 and 3). The simplest interpretation of the *pks4* loss- and gain-of-function mutants during the control of hypocotyl growth orientation suggests that PKS4 acts a negative regulator of asymmetric growth (Figs. 4–9). The genetic interaction between *pks4*, *phyA*, and *phyB* shows that the effect of PKS4 on growth orientation depends on phyA in far-red light whereas in red light it apparently depends on phyA, phyB, and at least a third photoreceptor (likely a phytochrome; Fig. 8). Given that there is currently no available data for the role of phyC, phyD, or phyE for this response we do not know which other phytochrome may be involved in this response. The genetic relationship between the different members of the *PKS* gene family during phytochrome-mediated control of hypocotyl growth orientation remains to be established.

The role of the PKS genes in the phytochrome-mediated control of hypocotyl growth orientation (Figs. 4–10), taken together with the importance of the *PKS* genes during hypocotyl phototropism (Lariguet et al., 2006) as well as root photo- and gravitropism (Boccalandro et al., 2008) define this small gene family as important regulators of hypocotyl and root growth orientation in the light. Interestingly, while the PKS proteins are positive regulators of phototropism, PKS4 inhibits changes in growth orientation under red or far-red light. Finally our results demonstrate that the effect of red light on hypocotyl growth orientation is more complex than previously anticipated. Interestingly, in a recent publication the authors also concluded that there are different pathways controlling hypocotyl growth orientation in red light (Nagashima et al., 2008). Importantly, our data show that red light does not significantly impair the gravi-reorientation potential of etiolated hypocotyls whereas it does impair gravi-reorientation of hypocotyls germinating in red light.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plant growth conditions and determination of light conditions were performed as described previously (Lariguet et al., 2003). The following mutants used in this study were described elsewhere: *pks1-1*, *pks2-1*, *pks4-1*, *pks4-2*, all *pks* mutant combinations, *phyA-211*, and *phyB-9* (Lariguet et al., 2003). Double mutants between *pks4-1* and *phyA-211* or *phyB-9* were obtained by crossing. To genotype *pks4-1* T-DNA insertion lines, we used: JMRB1 5′-GCT CAT GAT CAG ATT GTC GTT TCC CGC CTT-3′ and CF329 5′-CTT GGG ACT CGT AGG ATT CA-3′ giving a 440-bp PCR. CF329 and CF262 5′-CAA TGG CGC AAA CTA CTG TC-3′ were used to identify wild-type *PKS4* locus, giving a 780-bp PCR product.

PKS4 overexpressing plants were obtained by transforming Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) seedlings with construct pIS007 that codes for the *PKS4* complementary DNA driven by the cauliflower mosaic virus 35S promoter. *PKS4* coding sequence flanked with *Bam*HI (catalog no. R0136S; New England Biolabs) sites was amplified by PCR (IS01 5′-GGA TCC ATG GCG CAA ACT ACT GTC AC-3′ and IS02 5′-GGA TCC TGG TAT CCA TCA TTG CCT TG-3′), the *Bam*HI digested product was ligated using T4 DNA ligase (M0202S; New England Biolabs) into *Bam*HI digested pCGN18 to yield pIS007. All PCR-generated constructs were verified by sequencing. Two single insertion lines expressing elevated levels of PKS4 RNA (data not shown) were selected for further analysis (IS07-5 and IS07-11).

Hypocotyl Length, Cotyledon Opening, and Determination of Growth Orientation

Hypocotyl length and cotyledon opening measurements were performed as described in Lariguet et al. (2003). To measure the growth orientation of hypocotyls, seeds were plated on square dishes containing one-half strength Murashige and Skoog (MS) medium (catalog no. M021.0010; Duchefa) with 0.8% (w/v) agar. For the experiment performed with oriented seeds, the sterilized seeds were positioned using a binocular loupe. Plated seeds were stratified by treating them for 3 d at 4°C in darkness. Germination was induced by 1 h white light 100 μmol m^{-2} s^{-1}, and plates were placed vertically in chosen light condition at 22°C. Plates were scanned after 4 to 5 d growth and the angle of seedling relative to vertical was measured using ImageJ program. In the case of growth in darkness prior light exposure, the angle between the apical part of the hypocotyl and vertical was measured. Data

from the fluence rate response curves shown on Figure 7 were used to determine the fluence rates at which the other experiments were performed.

Time-Lapse Monitoring of Hypocotyl Orientation

The sterilized seeds were sown on agar plates (one-half strength MS with 0.8% [w/v] agar) and kept at 4°C for 3 d. Plates were subsequently transferred at 21°C ± 1°C and exposed to white light for 6 h to induce seed germination. Time-lapse images were acquired by using a CCD camera system composed of a binocular microscope (Nikon), monochrome CCD camera (CV-M50IR; JAI Japan), and infrared light-emitting diodes (FQ15603; Adlos AG; peak emission at 940 nm, half-bandwidth 50 nm) placed in an incubator (floraLED^S; CLF PlantClimatics GmbH). The MetaMorph software (Molecular Devices) was used to control the CCD camera system and to process images. The agar plates were set vertically and images were acquired every 30 min under darkness or in red-light condition at 21°C ± 1°C. To determine gravi-reorientation following 90° rotation of the seedlings we used stacked images (using National Institutes of Health ImageJ software version 1.38 [http://rsb.info.nih.gov/ij/]) as described by Folta et al. (2003). We selected seedlings that measured 4 to 5 mm at the time of reorientation and had cotyledons facing down after the 90° rotation because the position of the cotyledons has a significant impact on the tropic response (Khurana et al., 1989).

GUS Staining

Col-0 plants were transformed with construct pIS35. An *Spe*I genomic fragment obtained from BAC F21E1 was cloned into pBluescript II (Stratagene), giving the construct pIS13. The *Spe*I-*Nhe*I fragment of pIS13 was then cloned into pPCB308 (Xiang et al., 1999), giving construct pIS35. Hence, in pIS35, the 3,800-bp promoter region of *PKS4* drives the expression of 312 bp of the *PKS4* coding region fused in frame with *GUS*. Several independent transgenic lines were selected for their resistance to Basta and analyzed by GUS staining. The staining procedure was performed as described in Lariguet et al. (2003).

Materials and methods for the supplemental figures can be found online as Supplemental Data S1.

Supplemental Data

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

Supplemental Figure S1. PKS4 interacts with phyA and phyB.

Supplemental Figure S2. Light regulation of *PKS4* mRNA.

Supplemental Data S1. Supplemental materials and methods.

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