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# Reduced phototropism in pks mutants may be due to altered auxin-regulated gene expression or reduced lateral auxin transport.

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Running title: The role of PKS proteins in phototropism

**Summary**

Phototropism allows plants to orient their photosynthetic organs towards the light. In Arabidopsis, phototropins 1 and 2 sense directional blue light with phot1 triggering phototropism in response to low fluence rates, while both phot1 and phot2 mediate this response in higher light. Phototropism results from asymmetric growth in the hypocotyl elongation zone that depends on an auxin gradient across the embryonic stem. How phototropin activation leads to this growth response is still poorly understood. Members of the Phytochrome Kinase Substrate (PKS) family may act early in this pathway because PKS1, PKS2 and PKS4 are needed for a normal phototropic response and they associate with phot1 *in vivo*. Here we show that PKS proteins are needed both for phot1 and phot2-mediated phototropism. The phototropic response is conditioned by the developmental asymmetry of dicotyledonous seedlings with a faster growth reorientation when the cotyledons face away from the light compared to seedlings with the cotyledons facing the light. The molecular basis for this developmental effect on phototropism is unknown, here we show that PKS proteins play a role at the interface between development and phototropism. Moreover we present evidence for a role of *PKS* genes in hypocotyl gravi-reorientation that is independent of photoreceptors. *pks* mutants have normal levels of auxin and normal polar auxin transport however they show altered expression patterns of auxin marker genes. This suggests that PKS proteins control auxin signaling and/or are involved in lateral auxin redistribution.

## Introduction

As sessile photoautotrophic organisms plants constantly need to adapt their growth, physiology and development to the environment with fluctuating light conditions having a particularly strong influence on their entire life cycle. Higher plants sense such changes with multiple photoreceptors including a UV-B sensor, red and far-red photoreceptors of the phytochrome class and three distinct families of specific blue-light receptors namely the cryptochromes, phototropins and ZTL/FKF1/LKP2 (Kami *et al.* 2010, Rizzini *et al.* 2011). These photoreceptors modulate plant growth and development from seed germination until senescence by controlling the timing of key developmental transitions and initiating important adaptations (e.g. phototropism, shade avoidance) (Christie 2007, Franklin and Quail 2010, Kami*, et al.* 2010). Light sensing by the phototropins (phot1 and phot2 in *Arabidopsis thaliana*) allows optimization of photosynthetic activity by controlling a range of physiological responses including phototropism, leaf positioning, leaf flattening, chloroplast movements and opening of stomata (Christie 2007).

Phototropins are light-activated Ser/Thr protein kinases composed of an amino-terminal photosensory domain and a carboxy-terminal protein kinase domain (Christie 2007, Tokutomi *et al.* 2008). Two LOV (Light Oxygen Voltage) domains LOV1 and LOV2, each binding an FMN chromophore compose the light-sensing portion of the photoreceptor with LOV2 playing a particularly important role (Cho *et al.* 2007, Christie *et al.* 2002). Upon light perception the protein kinase domain is liberated from the inhibitory activity of the photosensory domain following a suite of light-induced

conformational changes (Harper *et al.* 2003, Matsuoka and Tokutomi 2005, Tokutomi*, et al.* 2008). In Arabidopsis several blue-light induced phosphorylation sites of phot1 and phot2 have been identified and it has been shown that phosphorylation in the activation loop of the protein kinase domain is essential for all tested physiological responses (Inoue *et al.* 2008a, Inoue *et al.* 2011, Sullivan *et al.* 2008). However, surprisingly little is known about the substrates of the phototropins (Christie *et al.* 2011, Demarsy *et al.* 2012).

Signal transduction events occurring upon phototropin activation depend at least partially upon the physiological response because several phototropin signaling elements are only required for a subset of phot-mediated responses (de Carbonnel *et al.* 2010, Inada *et al.* 2004, Inoue *et al.* 2008b). During phototropism NPH3 plays a particularly important function because a loss-of-function mutant is aphototropic in all tested conditions (Motchoulski and Liscum 1999). NPH3 and phot1 are plasma membrane-associated proteins interacting with each other (Lariguet *et al.* 2006, Motchoulski and Liscum 1999, Sakamoto and Briggs 2002). In rice CPT1, the ortholog of NPH3, is essential for phototropism and acts upstream of lateral auxin redistribution that is needed for a phototropic response (Esmon *et al.* 2006, Haga *et al.* 2005). The interaction between phot1 and NPH3 and its requirement upstream of auxin redistribution indicates that NPH3 acts early during phototropism signaling. NPH3 is rapidly dephosphorylated in response to blue light in a phot1-dependent manner (Pedmale and Liscum 2007), moreover it is required for the down-regulation of phot1 protein in the light (Roberts *et al.* 2011). Both auxin transport and auxin signaling are required downstream of these

early events (Christie*, et al.* 2011, Ding *et al.* 2011, Stone *et al.* 2008, Tatematsu *et al.* 2004, Willige *et al.* 2013).

Members of the PKS (Phytochrome Kinase Substrate) family (PKS1-PKS4 in Arabidopsis) play a role in a subset of phototropin-mediated responses (Boccalandro *et al.* 2008, de Carbonnel*, et al.* 2010, Lariguet*, et al.* 2006, Zhao *et al.* 2013). PKS1, PKS2 and PKS4 localize to the plasma membrane where they are associated with phot1, phot2 and NPH3 (de Carbonnel*, et al.* 2010, Demarsy *et al.* 2012, Lariguet*, et al.* 2006). PKS4 is phosphorylated by phot1 within seconds of blue light perception (Demarsy*, et al.* 2012). Taken together with the strong genetic interactions between *nph3* and *pks2* (de Carbonnel*, et al.* 2010), this suggests that PKS proteins may also act early in phototropin signaling. However how they control phototropism remains unknown. An additional complication comes from the role that PKS proteins also play in phytochrome signaling (Lariguet *et al.* 2003, Schepens *et al.* 2008). Importantly, phytochromes and in particular phyA enhance phototropism and this may at least in part depend on phyA-mediated induction of *PKS1* expression (Kami *et al.* 2012, Lariguet*, et al.* 2006).

To obtain further insight into the role of *PKS* genes in phototropism, we characterized the phototropic response of *pks1*, *pks2* and *pks4* single, double and triple mutants grown under different blue light intensities. Using higher order mutants between *pks* and *phot* or *phyA* we showed genetically that PKS proteins primarily act in phot1 signaling. Tropic responses of the hypocotyl are developmentally modulated with the orientation of the cotyledons relative to the incoming light influencing the response (Khurana *et al.* 1989).

Our analysis shows that PKSs are important for this developmental regulation of the tropic response. Mechanistically we show that PKS proteins are dispensable for plasma membrane localization of phot1. Auxin levels and polar auxin transport are normal in *pks1pks2pks4* seedlings. However, auxin-regulated gene expression in the etiolated hypocotyl hook region is altered in *pks1pks2pks4*. We propose that PKS proteins act early in phot1 signaling by modulating auxin signaling and/or lateral transport.

## Results

In a previous study, we showed that *pks1*, *pks2* and *pks4* are required for a normal phototropic response (Lariguet*, et al.* 2006). Our results suggested that PKS proteins act in the phot1 rather than the phyA pathway in the control of phototropism but this was not formally demonstrated. In order to do so we first analyzed phototropism in response to different fluence rates of unilateral blue light using long-term phototropic assays (Lariguet and Fankhauser 2004). Consistent with previous experiments, *phyA* mutants showed a reduced phototropic response most strikingly under low fluence rates (0.1 μmoles $m^{-2}$ $s^{-1}$ and below), while *phot1* was defective at all fluence rates tested (Figure S1) (Kami*, et al.* 2012). In agreement with the notion that PKS proteins act in the phot1 pathway, the phenotype of *pks1pks2pks4* triple mutant was more similar to the phenotype of *phot1* than the phenotype of *phyA* (Figures 1, S1). To test this hypothesis genetically we made *phyApks1pks2pks4* and *phot1pks1pks2pks4* mutants that we compared to the wild type, *phyA*, *phot1*, *pks1pks2pks4* and *phyAphot1*. We used "long-term" phototropism assays to characterize those mutants because under these conditions it is very easy to distinguish *phyA* from the *pks1pks2pks4* triple mutant (de Carbonnel*, et al.* 2010, Demarsy*, et al.* 2012, Lariguet*, et al.* 2006) (Figures 1, S1). If PKS1, PKS2 and PKS4 acted in the phot1 pathway the *phot1pks* quadruple mutant would be expected to look like *phot1*. We observed that the *phot1pks* quadruple mutant looked very similar to *phot1* (Figure 1, S2). Moreover, *phyApks1pks2pks4* and *phot1phyA* behaved very similarly under these conditions (Figure 1) (Lariguet and Fankhauser 2004). Statistical analysis of the recorded growth orientations confirmed this visual assessment (Figure 1B).

Collectively these data indicate that in low blue light PKS1, PKS2 and PKS4 act in the phot1 pathway to control phototropism.

Next we analyzed phototropism in etiolated seedlings exposed for 24 hours to unilateral blue light of different fluence rates (0.1, 1 and 10 □moles $m^{-2}$ $s^{-1}$) to analyze both phot1 and phot2-mediated phototropism (Sakai *et al.* 2001). As observed previously, *phot1* showed no phototropic response at the lower fluence rates while it still responded partially to 10 □moles $m^{-2}$ $s^{-1}$, a response that was largely abolished in *phot1phot2* (Figure 2) (Sakai*, et al.* 2001). The analysis of *pks* single mutants allowed us to determine that only *pks4* showed significantly reduced phototropism but specifically at the lowest fluence rates tested (Figure S1 and S3). Among all double mutants *pks1pks4* showed the strongest phenotype at the lower fluence rates while *pks1pks2* showed the strongest phenotype at the highest fluence rate (Figure 1C). The *pks1pks2pks4* triple mutant behaved as *pks1pks4* at the lower fluence rates and as *pks1pks2* at the highest fluence rate (Figure 1C). These results suggested that in particular PKS2 and PKS1 may also play a role in phot2-mediated phototropism occurring in response to higher fluence rates. In agreement with this hypothesis the *phot1pks1pks2pks4* quadruple mutant was totally defective in phototropism in response to high blue light (Figure 1D). Although this genotype appears more affected than *phot1phot2*, this difference was not significant ($P>0.001$). In summary these data indicate that PKS4 is primarily important for phototropism in response to low light, PKS2 in response to strong light and PKS1 is needed under all light conditions additively with PKS4 or PKS2 depending on light intensity. Moreover PKS proteins act both in phot1 and phot2-mediated phototropism.

Finally our genetic analysis showed that a weak phototropic response still occurred in the *pks1pks2pks4* triple mutant. We thus decided to compare the phenotype of *pks1pks2pks4* with the other mutants that were previously shown to have a reduced phototropic response (Christie*, et al.* 2011, Ding*, et al.* 2011, Stone*, et al.* 2008, Tatematsu*, et al.* 2004). Our experiments showed that in two different experimental conditions the phototropic response in *pks1pks2pks4* was weaker than in *iaa19/msg2* and *pin3* mutants (Figure 1 and S2).

The molecular function of PKS proteins is currently unknown. PKS1, PKS2 and PKS4 are all associated with the plasma membrane similarly to the phototropins (de Carbonnel*, et al.* 2010, Demarsy*, et al.* 2012, Lariguet*, et al.* 2006). How phot1 associates with the membrane is poorly understood (Kong *et al.* 2013), we thus tested whether PKS proteins were needed for proper phot1-GFP localization by crossing plants expressing this transgene (Sakamoto and Briggs 2002) with *pks1pks4* double mutants and selecting *pks1pks4phot1PHOT1:PHOT1-GFP* seedlings. We analyzed phot1-GFP localization in *pks1pks4* because under low fluence rates of blue light because PKS1 and PKS4 play a primary role in phot1-mediated phototropism (Figure 1C). Phot1-GFP associates with the plasma membrane in etiolated seedlings while a portion of the photoreceptor dissociates from the membrane in response to blue light (Sakamoto and Briggs 2002). Using maximal projection images the dissociation of phot1-GFP from the plasma membrane results in a non-homogeneous GFP signal after the light treatment (Wan *et al.* 2008) (Figure 2). The analysis of phot1-GFP in the wild type and *pks1pks4* backgrounds revealed a normal phot1-GFP localization in the mutant both in darkness and in response

to blue light (Figure 2). We thus conclude that PKS proteins do not modulate phototropism by controlling phot1 subcellular localization.

We identified a unique aspect of the *pks* phototropic phenotype by using time course experiments (Figure 3). We separately analyzed phototropism of seedlings, which had their cotyledons towards or away from the incoming light because cotyledon position was previously shown to affect the phototropic response (Kami*, et al.* 2012, Khurana*, et al.* 1989). The effect of cotyledon position on phototropism decreases with increasing fluence rates (Figure S5). At the fluence rate used for our experiment cotyledon position had a small effect on phototropism of the wild type and of *phyA*, *msg2* and *pin3* mutants (Figure 3). In contrast both in *pks1pks4* and in *pks1pks2pks4* there was a very large difference in phototropism between the seedlings having their cotyledons towards or away from the light (Figure 3).

Cotyledon position also influences the gravitropic response in etiolated hypocotyls (Khurana*, et al.* 1989). We thus compared gravitropism in etiolated wild type and *pks1pks2pks4* mutants and monitored the response of seedlings with cotyledons facing up and down separately (Figures 4, S6 and S7). As reported previously when cotyledons faced downwards gravitropism occurred much more efficiently in the wild type (Figure 4) (Khurana*, et al.* 1989). In contrast in the *pks1pks2pks4* triple mutant the position of the cotyledons had no influence on the gravitropic response (Figure 4). As PKS proteins act both in the phyA or the phot pathway depending on the conditions (Figure 1) (Lariguet*, et al.* 2003, Schepens*, et al.* 2008), we also tested this response in *phyA*, *phot1*, *phot1phot2*,

*phyApks1pks2pks4* and *phot1pks1pks2pks4* mutants (Figure 4 and S7). Interestingly, both *phyA* and the phototropin mutants showed a diminished ability to re-orient their hypocotyl upon gravitropism in darkness (Figures 4 and S7). Moreover, in these photoreceptor mutants we observed no significant difference in the gravitropic response between seedlings with their cotyledons facing up or down at the time of gravistimulation (Figures 4 and S7). Finally the *phyApks1pks2pks4* and *phot1pks1pks2pks4* mutants showed a phenotype that is intermediate between the photoreceptor and *pks* mutant lines (Figures 4 and S7). These data suggest that *PKS* genes play a role independent of the photoreceptors in etiolated seedlings. We also analyzed hypocotyl gravitropism in the light. In order to prevent a phototropic response we analyzed gravitropism in etiolated seedlings transferred into red light. As shown previously this lead to an altered gravitropic response in the wild type (Nagashima *et al.* 2008) (Figure S6). Our results show that this altered response was most clearly visible when analyzing seedlings with cotyledons facing up and down separately (Figure S6). Interestingly, light had a stronger effect on the gravitropic response of the wild type than *pks1pks2pks4* mutants (Figure S6).

Etiolated seedlings show a *DR5* gradient in the hypocotyl hook suggesting the presence an auxin signaling gradient in the uppermost portion of the hypocotyl (Zadnikova *et al.* 2010). The influence of cotyledon position on the gravitropic and phototropic response of wild type seedlings may thus be related to this presumptive auxin gradient in the hook region of the hypocotyl. *pks* mutants show an altered phototropic and gravitropic response (Figures 1, 3, 4) (Lariguet*, et al.* 2006), moreover the effect of cotyledon

position on these tropic responses is largely modified in *pks1pks2pks4* (Figures 3, 4 and S6). We thus decided to analyze auxin content, polar auxin transport and auxin signaling in the *pks1pks2pks4* mutant. Auxin content of aerial parts of the wild type and *pks1pks2pks4* was determined both in etiolated seedlings and etiolated seedlings treated for 8 hours with light. This experiment confirmed the light-induced decline in aerial auxin content reported previously (Nagashima*, et al.* 2008) and showed that auxin content was normal in *pks1pks2pks4* in both experimental conditions (Figure 5A). Polar auxin transport (PAT) was determined in etiolated seedlings and etiolated seedlings treated with light for 1, 2 or 4 hours of light (Willige *et al.* 2013). NPA treatment largely inhibited PAT in etiolated wild-type hypocotyls (Figure 5B). Over the time course of our experiment we did not detect any significant effect of light on PAT. Finally PAT was normal in etiolated and light treated *pks1pks2pks4* seedlings (Figure 5B).

*DR5:GUS* and *MSG2:GUS* were used as reporters to analyze auxin signaling. These constructs were crossed into *pks1pks2pks4* triple mutants and reporter activity was compared in etiolated wild-type and *pks* mutant seedlings (Figure 6). We concentrated our analysis on this developmental stage because etiolated *pks1pks2pks4* mutants have normal auxin levels and PAT thereby simplifying the interpretation of auxin reporter activity (Figure 5). Moreover, the *pks1pks2pks4* mutant shows a phenotype very early upon photostimulation and has an altered response upon gravity induced re-orientation in darkness (Figures 3, 4 and S6). As reported previously strong *DR5* activity was observed on the concave side of the apical hook (Zadnikova*, et al.* 2010) (Figure 6). Interestingly this gradient was largely absent in *pks1pks2pks4* triple mutants (Figure 6A and B). This is

not due to an overall decrease in *DR5* signal in the mutant as both the wild type and *pks1pks2pks4* cotyledons show a strong *DR5* driven GUS expression (Figure 6A and B). The signal for *MSG2:GUS* was also strikingly altered in the *pks1pks2pks4* mutant. First, the signal just below the hook region was reduced in the mutant, a reduction that was quantified in transverse sections (Figure 6A and B). In addition the expression along the length of the hypocotyl was also altered in the mutant. In the wild type there was strong staining at the top of the hypocotyl, the activity declined quite sharply reaching a minimum around 35-40% down from the tip followed by a rapid increase towards the base of the hypocotyl (Figure 6 C and D). In *pks1pks2pks4* the decline from top to the middle third was more shallow and the increase in staining towards to lower part of the hypocotyl occurred later than in the wild type (Figures 6 C and D). Thus, both reporters indicate that the pattern of auxin responsive genes was altered in *pks1pks2pks4* hypocotyls, a feature that is particularly striking in its uppermost part (Figure 6).

## Discussion

Our systematic characterization of *pks* mutant combinations with *phot1*, *phot2* and *phyA* allowed us to determine that *PKS* genes are important for phot1-mediated phototropism (Figures 1, S1-S3). In addition our data reveal that in response to high fluence rates of blue light PKS proteins also act in the phot2 pathway which is consistent with a recent report (Figure 1) (Zhao*, et al.* 2013). We previously showed that PKS1 and PKS2 primarily act in the phot1 pathway in the control of petiole positioning while they primarily act in the phot2 pathway in the control of leaf flattening (de Carbonnel*, et al.* 2010). Here we show that PKS proteins act both in the phot1 and the phot2 pathway in the control of a single physiological response (phototropism). Our extensive genetic and photobiological analysis identified a differential requirement for the members of the *PKS* family in response to different light conditions (Figure 1C, D). PKS4 is primarily important in response to low fluence rates, PKS2 is mostly required in high light while PKS1 is required in all light conditions (Figure 1 and S3). The conditional phenotype of the *pks* mutants correlates well with light-regulated *PKS* gene expression. *PKS4* levels decline in etiolated seedlings transferred into the light while *PKS2* expression is induced by light (Lariguet*, et al.* 2003, Schepens*, et al.* 2008). The *pks1pks2pks4* triple mutant is strongly defective for phototropism with only a minor response towards low blue light fluence rates (Figures 1 and 3). However in contrast to *phot1phot2* the *pks1pks2pks4* still displays a phototropic response particularly in response to high blue light fluence rates (Figure 1). Once a mutant in *PKS3* becomes available it will be interesting to determine the behavior of a *pks* quadruple mutant.

Both phot1 and members of the PKS family localize to the plasma membrane (de Carbonnel, *et al.* 2010, Lariguet, *et al.* 2006, Sakamoto and Briggs 2002). It has been shown that Brefeldin A (BFA) inhibits phot1 localization suggesting that its export to the plasma membrane occurs via the trans-Golgi network (Kaiserli *et al.* 2009). Upon blue light perception a fraction of phot1 internalizes a process that is mediated through the clathrin-dependent endocytic pathway (Kaiserli, *et al.* 2009, Sakamoto and Briggs 2002). However the mechanism underlying phot1 association with the plasma membrane is not well understood because the receptor does not have any transmembrane or known membrane association domains (Kong *et al.* 2013). One possibility is that phot1 binds to the plasma membrane via protein-protein interactions. Based on their subcellular localization, their ability to interact with phot1 and their importance for phototropism PKS proteins are good candidates for such a function (de Carbonnel, *et al.* 2010, Demarsy, *et al.* 2012, Lariguet, *et al.* 2006). However, our microscopic examination of etiolated and light-treated seedlings did not reveal any significant difference in the localization of phot1-GFP in *pks1pks4* compared to the wild type indicating that PKS proteins are not needed for normal subcellular localization of phot1 (Figure 2).

A striking feature of the *pks* phototropic and gravitropic phenotype is the strongly altered developmental regulation of the response in the mutants (Figures 3 and 4). Both the gravitropic and the phototropic responses are modulated by the position of the cotyledons relative to the direction of the stimulus (Khurana, *et al.* 1989). Phototropism is more effective when the cotyledons are away from the incoming light while gravitropism is

more effective when cotyledons face downwards (Khurana*, et al.* 1989) (Figures 3, 4 and S6). Cotyledon positioning affects phototropism mostly in response to low light, moreover cotyledon position primarily alters the kinetics of the response rather than the final bending angle (Figures 3 and S5) (Kami*, et al.* 2012). The *pks1pks4* and *pks1pks2pks4* mutants had a unique phenotype during phototropism with a difference in bending kinetics depending on cotyledon position that was larger than in the wild type and various mutants that we have tested (*phyA*, *msg2* and *pin3*). Interestingly *pks* mutants with their cotyledons facing the incoming light are almost aphototropic, while when the cotyledons are on the shaded side these mutants display a phototropic response (Figure 3). The effect of cotyledon position on the hypocotyl gravitropic response was also strikingly altered in *pks1pks2pks4* hypocotyls (Figure 4 and S6). However, under these conditions the wild type showed a large dependency on cotyledon position for the gravitropic response, while in *pks1pks2pks4* gravitropism occurred with the same kinetics irrespective of cotyledon position. Thus compared to the wild type the *pks1pks2pks4* mutants display a bigger bending difference depending on cotyledon position during phototropism but a smaller difference during gravitropism (Figures 3 and 4). This data indicates that the *pks1pks2pks4* mutant is not simply unable to display a different growth response depending on the position of its cotyledons but the coupling between morphology and response to stimulus is altered. In addition, our work shows that in some conditions (e.g. darkness) *PKS* genes play a role in asymmetric growth responses that is independent of the action of photoreceptors indicating a broad importance of *PKS* genes in asymmetric growth responses (Figures 4 and S7).

The molecular mechanism underlying the effect of cotyledon position on phototropic and gravitropic bending is unknown. One hypothesis is that it is due to the presumptive auxin-signaling gradient present in the apical hook (Zadnikova*, et al.* 2010). If one assumes higher auxin signaling on the hypocotyl side facing the cotyledons (based on the stronger *DR5:GUS* signal), this would explain why seedlings having their cotyledons away from unilateral light bend faster towards the light because the "developmental auxin gradient" is already in the right position. Although during phototropism asymmetric growth occurs below the hook region it was recently suggested that an auxin gradient in the hypocotyl just below the cotyledons is important for phototropism (Christie*, et al.* 2011). In contrast, when cotyledons face the light this presumptive auxin maximum would have to be displaced to the other side of the hypocotyl to allow differential growth towards the light source. Under these conditions *pks1pks4* and *pks1pks2pks4* are very strongly impaired in the phototropic response suggesting that PKS proteins may be required for lateral auxin transport or act upstream of this process (Figure 3). Interestingly when the auxin gradient is favorably positioned (cotyledons face away from the incoming light), the defect of *pks* mutants is significantly weaker (Figure 3).

The same reasoning could also explain the effect of cotyledon position on the gravitropic response because hypocotyls with their cotyledons facing down show a more rapid gravitropic response and based on the concept of the "developmental auxin gradient" more auxin signaling would then initially be on the side that grows faster in order to reorient hypocotyls with the gravity vector (Khurana*, et al.* 1989). The tropic phenotype of *pks1pks2pks4* is condition/stimulus dependent as in contrast to phototropism, the

mutant displays enhanced gravitropism and a reduced effect of cotyledon position on gravitropism (Figures 3 and 4). Interestingly, the absence of effect of cotyledon position during gravtitropism correlates with the absence of a *DR5:GUS* gradient in the hook region of etiolated *pks1pks2pks4*. Taken together the results suggest that PKS proteins act as a control point at the interface between development and hypocotyl response to external stimuli.

Defects in gravitropism and phototropism could be due to altered auxin content or reduced polar auxin transport that can lead to auxin depletion in the hypocotyl elongation zone (Christie 2007, Willige*, et al.* 2013). Determination of auxin content in aerial parts of *pks1pks2pks4* shows that this mutant has free IAA levels that are not significantly different from the wild type both in etiolated seedlings and etiolated seedlings treated with light for a few hours (Figure 5). In addition polar auxin transport in the hypocotyl is normal in etiolated *pks1pks2pks4* seedlings before and after a light treatment (Figure 5). This distinguishes the *pks1pks2pks4* phenotype from a *pin3pin4pin7* mutant that shows reduced polar auxin transport (Willige*, et al.* 2013). Interestingly, both mutants are strongly impaired for phototropism, however *pks1pks2pks4* hypocotyls grow against the gravity vector in darkness while *pin3pin4pin7* do not (Figure S1) (Lariguet*, et al.* 2006, Willige*, et al.* 2013). We thus conclude that while the hypocotyl tropic phenotypes of *pin3pin4pin7* could at least in part be due to reduced levels of auxin in the hypocotyl elongation zone (due to reduced PAT) this is unlikely to be the case for *pks1pks2pks4* mutants.

To analyze the auxin response in this mutant we used *DR5:GUS* and *MSG2:GUS* as reporter constructs. In etiolated *pks1pks2pks4* seedlings the *DR5:GUS* signal was significantly altered in the apical hook and just below (Figure 6). While a distinct *DR5:GUS* gradient can be measured in the wild type this was not the case in the mutant. Moreover, the levels of *MSG2:GUS* in the hook region and just below are also significantly reduced in *pks1pks2pks4*. Finally the pattern of *MSG2:GUS* expression along the hypocotyl longitudinal axis was also altered in *pks1pks2pks4* mutants. *DR5* marker genes have been used to analyze lateral auxin gradients in the hypocotyl. However, it was recently shown that the *DR5* gradient induced by phototropic stimulation is only visible after phototropic bending was over (Haga and Sakai 2012). Similarly we have not observed any *MSG2:GUS* gradient across the hypocotyl of photostimulated hypocotyls (data not shown). To the best of our knowledge there currently is no reliable marker allowing us to detect an auxin gradient across the hypocotyl that precedes phototropic bending in etiolated seedlings.

Given that *pks1pks2pks4* mutants have normal IAA levels in the aerial parts and normal polar auxin transport in the hypocotyl (Figure 5), we propose that *PKS* genes are essential for a normal response to auxin in the hypocotyl (Figure 6). Although auxin signaling is altered in *pks1pks2pks4* (Figure 6), this mutant has a clearly distinct phenotype from other auxin signaling mutants such as *msg2* (Figures 1 and 3) (Tatematsu*, et al.* 2004; Schepens*, et al.* 2008). The *msg2* mutant shows reduced negative hypocotyl gravitropism in darkness, reduced gravi-reorientation and reduced phototropism (Tatematsu*, et al.* 2004). In contrast *pks1pks2pks4* show normal negative hypocotyl gravitropism in

darkness, an enhanced gravi-reorientation but reduced phototropism (Figures 3, 4 and S6) (Schepens*, et al.* 2008). Moreover the phototropic dependency on cotyledon position is strongly enhanced in *pks1pks2pks4* while it is similar to the wild type in *msg2* (Figure 3). The phenotype of *pks1pks2pks4* mutants is therefore clearly distinct from typical auxin signaling or auxin transport mutants. We propose that PKS proteins are either required for the establishment of a local lateral auxin gradient or for the response to this gradient.

## Experimental Procedures

### Plant material and growth conditions

Plant growth conditions, determination of light conditions were performed as described previously (Kami*, et al.* 2012). The following mutants used in this study were described elsewhere: *msg2-1* (Tatematsu*, et al.* 2004), *pin3-3* (Friml *et al.* 2002), *pks1-1*, *pks2-1*, *pks4-1*, *pks4-2*, *phyA-211*, *phot1-5*, *phot2-1*, *phot1phot2*, all *pks* mutant combinations as well as *pksphot1*, *pksphot2* and *pksphyA* mutant combinations (de Carbonnel*, et al.* 2010) (Lariguet*, et al.* 2003) (Schepens*, et al.* 2008).

The *MSG2* promoter sequence was amplified by PCR using a pair of oligonucleotides, 5'-ATGGAGCTCGCGGCCGCGTTCCTTCGCATCGGATTTGACGAAGATC-3' and 5'-CATGAATTCGGGATCGATGTCGACTTCTTGAACTTCTTTTTTTCCTCTCACAAT-3', and the genomic DNA of Arabidopsis (Col-0) as a template. The resulting 3106-bp fragment was cloned into the SacI and XhoI sites of pART7 (Gleave 1992), which harboured beta-glucuronidase cDNA in BamHI and XbaI sites. The promoter-GUS construct was cloned into T-DNA vector, pART27 for Agrobacterium-mediated floral dip transformation (Clough and Bent 1998).

### Tropism assays

Long-term phototropism assays were performed as described previously (Lariguet and Fankhauser 2004). Short-term phototropism, kinetic analysis of phototropism and

gravitropism assays were performed as described in (Kami, *et al.* 2012, Schepens, *et al.* 2008).

Determination of auxin content and polar auxin transport

Three-day-old etiolated seedlings were harvested under safe green light or treated for 8 hours with 20 □moles $m^{-2}$ $s^{-1}$ white light before harvest. Seedlings were pooled weighted and frozen in liquid nitrogen for quantification of free IAA according to (Andersen *et al.* 2008). Polar auxin transport (PAT) was determined as described in (Willige, *et al.* 2013). Etiolated Col (wild type) or *pks1pks2pks4* triple mutants were treated for 0, 1, 2 or 4 hours with blue light before determining PAT. As a control etiolated wild-type seedlings were treated with NPA (Willige, *et al.* 2013).

GUS staining

*DR5:GUS* and *MSG2:GUS* were crossed with *pks1pks2pks4* to obtain the reporter lines in the *pks* triple mutant. *pks* mutations were identified in the F2 as described previously (Lariguet, *et al.* 2003) (Schepens, *et al.* 2008). GUS hischemical analysis was performed as previously described but without fixation (Lariguet, *et al.* 2003). Three-day-old etiolated seedlings (4.0 - 5.9 mm) were incubated with GUS staining solution (2 mM 5-bromo-4chloro-3-indolyl B-D-glucuronide, 2 mM ferrocyanide and 50 mM sodium phosphate buffer) for 24 hours (*DR5:GUS*) or 6 hours (*MSG2:GUS*). Seedlings were observed and imaged with a binocular loop (Nikon SMZ1500, Tokyo, Japan). Images were processed with ImageJ (National Institutes of Health ImageJ software version 1.38

[http://rsb.info.nih.gov/ij/]). Image analysis was performed as previously described (Santuari *et al.* 2011).

Microscopy

Projection images of confocal microscopy and image analysis were performed essentially as previously described (Wan*, et al.* 2008) (Han *et al.* 2008). Three-day-old etiolated seedlings (4.0 - 5.9 mm) were placed on slides in a drop of half strength MS medium with 0.01% (w/v) agar. GFP fluorescence was imaged with an inverted Zeiss confocal microscope (LSM 510 Meta INVERTED, Zeiss AXIO Vert 200 M ; x40 objective) with the 488 nm excitation line of 30.0 mW argon ion laser and band-pass filters (excitation 405- 488 nm, emission 505- 550 nm). Images were processed with Zeiss software (LSM Rel. 3.5) and ImageJ (National Institutes of Health ImageJ software version 1.38 [http://rsb.info.nih.gov/ij/]).

Statistical analysis

Statistical computations were performed using the R software (http://www.r-project.org/), version 2.15.1. Clusters were produced using the default parameters (Euclidean distance, complete linkage). Difference between the distribution of values for different groups was measured using a Kolmogorov-Smirnov statistic (maximum difference between cumulative distributions). Its significance was assessed using permutation of the individual observations, assuming that the groups were identical. Over 5000 permutations, the measured statistics was never reached (p-value=1/5001 < 0.0002), indicated a significant difference between the groups. To compare phototropism of

different genotypes (Figures 1C, D) we used the Student's t-test (two tails distribution, two-sample unequal variance).

**Supplemental data**

Supplementary Figure S1. Comparison of hypocotyl growth orientation in *phyA, phot1, nph3* and *pks* mutants grown in constant darkness and unilateral blue light (long-term phototropic response).

Supplementary Figure S2. Long-term phototropic response of *pks* photoreceptor mutant combinations.

Supplementary Figure S3. Phototropic phenotype of etiolated *pks* mutants treated for 24 hours with different blue light fluence rates.

Supplementary Figure S4. Long-term phototropic response of auxin signaling, transport and *pks* mutants.

Supplementary Figure S5. Kinetics of the phototropic response in WT under low and high blue light

Supplementary Figure S6. Gravi-reorientation responses in WT and *pks1pks2pks4* in darkness and red light.

Supplementary Figure S7. Gravi-reorientation responses in WT, *phyA*, *phot1*, *phot1phot2*, *pks1pks2pks4*, *phyApks1pks2pks4* and *phot1pks1pks2pks4* mutants.

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**Figure legends**

Figure 1. *PKS* genes are important for phot1-mediated and phot2-mediated phototropism. (A) Long-term phototropism experiment. Seedlings were grown on vertical plates for 65 hour with unilateral blue light (0.1 μmoles $m^{-2}$ $s^{-1}$). Final growth angle relative to vertical was measured (0° represents vertical growth). The direction of growth is shown as circular histograms with the percentage of seedlings present in 10° angle categories (n>100). The scale is indicated in the top left corner. (B) Hierarchical clustering showing similarities between the profiles for the different groups. The "height" axis indicates the Euclidean distances between groups of profile. A permutation test based on a Kolmogorov-Smirnov statistics indicates a highly-significant difference ($p < 0.0002$) between the profiles for the two main branches of the tree. (C) and (D) Short-term phototropism experiment. Three-day-old etiolated seedlings (4.0 – 5.9 mm hypocotyls) were exposed to blue light (0.1 light grey, 1 intermediate grey or 10 dark grey μmoles $m^{-2}$ $s^{-1}$) for 24 hours. Data are average angles relative to vertical +/- 2X SE (n>95). a: different from the WT grown at 0.1 μmoles $m^{-2}$ $s^{-1}$ $P<0.001$, b: different from the WT grown at 1.0 μmoles $m^{-2}$ $s^{-1}$ $P<0.001$, c: different from the WT grown at 10 μmoles $m^{-2}$ $s^{-1}$ $P<0.001$.

Figure 2. PKS1 and PKS4 are not required for phot1 relocalization under low blue light. Maximal projection images of phot1:GFP in the *phot1* or *phot1pks1pks4* background. Three-day-old etiolated seedlings (4.0 – 5.9 mm) were scanned (15 section at 1 μm depth intervals) four times with 5min darkness between each scan. Confocal microscopy

images were obtained with 488nm laser (1 μmoles $m^{-2}$ $s^{-1}$, 120 seconds scanning time for the 15 sections). Bars = 30 μm.

Figure 3. Kinetics of the phototropic response in WT, *phyA, pks1pks4, pks1pks2pks4, msg2* and *pin3*. Dark-grown seedlings (4.0-5.9 mm) were exposed to unilateral blue light (0.5 μmoles $m^{-2}$ $s^{-1}$) for 5 hours. Growth angle of hypocotyls relative to vertical were measured (0° represents vertical growth).

(A) Data are average (full circle) hypocotyl angles from both cotyledon positions (n= 34-40 with 17-20 for each cotyledon position) +/- 2X SE.

(B) Data are average hypocotyl angles for seedlings in H position (hypocotyl towards the light, full triangle) and C position (cotyledon towards the light, open triangle) shown separately. Data are average +/- 2X SE (n= 17-20, each position).

Figure 4. Gravi-reorientation response in WT, photoreceptor mutants and *pks1pks2pks4.* Three-day-old (4.0 – 5.9 mm) etiolated seedlings of the indicated genotypes were grown on vertical plates. The plates were rotated 90°, and images acquired hourly for 24 hours in darkness. Gravitropic reorientation with different cotyledon positions (d: cotyledons downwards shown as full circles, u: cotyledons upwards shown as open circles). Data are average hypocotyl angles from 20 hypocotyls +/- 2X SE.

Figure 5. IAA concentration and polar auxin transport in *pks1,2,4* triple mutants.

A: Auxin content of etiolated seedlings (dark) or etiolated seedlings treated for 8 hours with light (20 μmoles $m^{-2}$ $s^{-1}$). Data are average of n=5 +/- 2X SE. B: Polar auxin

transport in etiolated Col (WT) and *pks1 pks2 pks4* triple mutants (*pks1, 2, 4*) treated for 0, 1, 2 and 4 hours of light. Data are average of n=8 +/- SE.

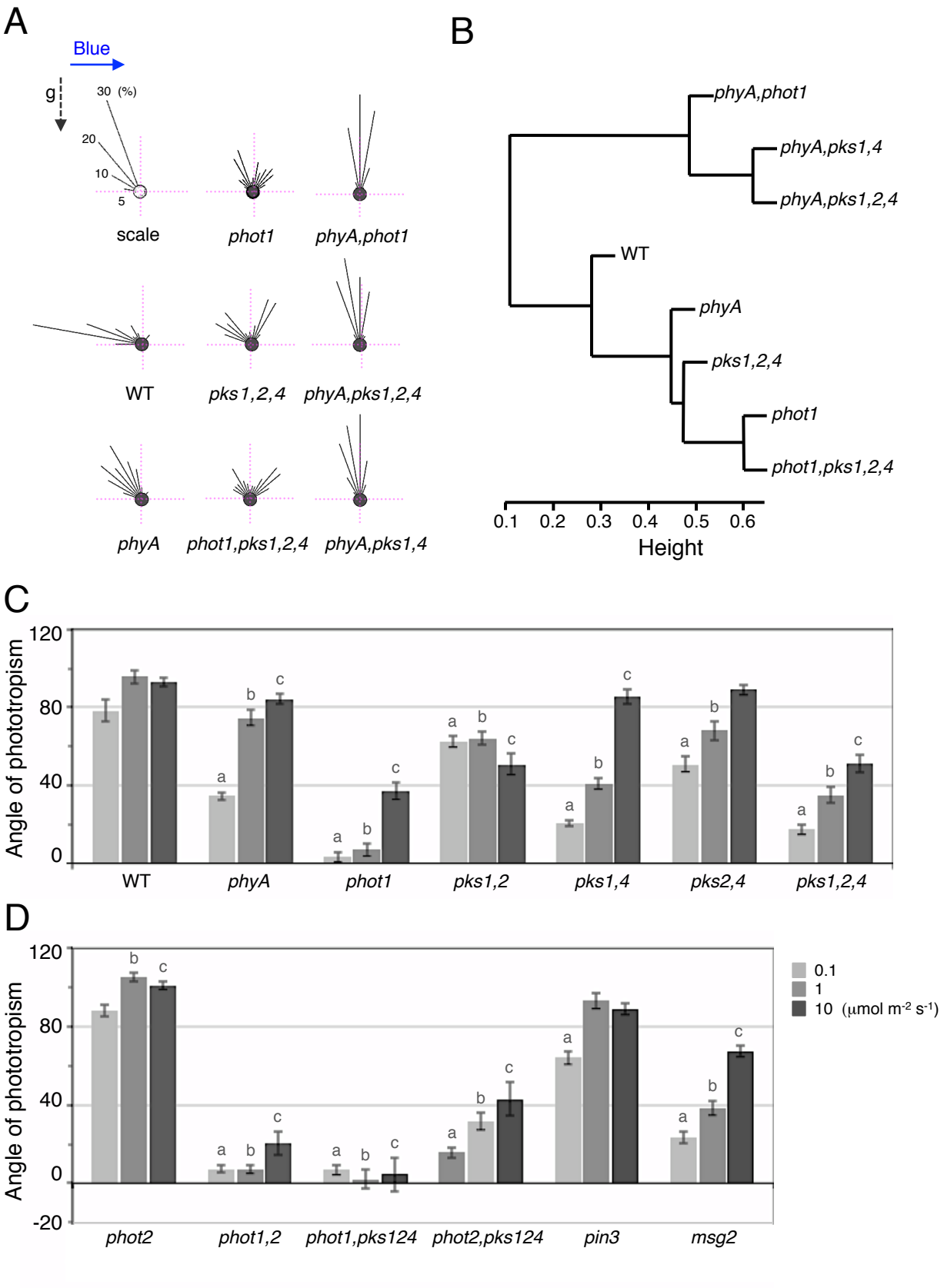
Figure 6. Auxin response is altered in *pks1pks2pks4* mutants. A: Comparison of *DR5:GUS* or *MSG2:GUS* expression between WT and *pks1pks2pks4*. Bar = 250 μm.

B: Normalized GUS stain intensity in elongation zone. Data are average intensity with +/- 2X SE of transverse section from inside of hook to outside (WT: full circle, *pks1,2,4*: open triangle, n=10). The orange arrow indicates the position of the section.

C: Comparison of MSG2:GUS expression between WT and *pks1,2,4*. Bar = 1mm.

D: Normalized GUS stain intensity in hypocotyl. Data are average intensity with +/- 2X SE of longitudinal section from the inside of the hook side from the hook to end of the hypocotyl (WT: full circle, *pks1,2,4*: open triangle, n=10).

Figure 1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Height
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Angle of phototropism
120
80
40
0
WT
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phot1
pks1,2
pks1,4
pks2,4
pks1,2,4
D
Angle of phototropism
120
80
40
0
-20
phot2
phot1,2
phot1,pks124
phot2,pks124
pin3
msg2
0.1
1
10 (μmol m-2 s-1)

Figure 1. *PKS* genes are important for phot1-mediated and phot2-mediated phototropism.
(A) Long-term phototropism experiment. Seedlings were grown on vertical plates for 65 hour with unilateral blue light (0.1 μmoles $m^{-2}$ $s^{-1}$). Final growth angle relative to vertical was measured (0° represents vertical growth). The direction of growth is shown as circular histograms with the percentage of seedlings present in 10° angle categories (n>100). The scale is indicated in the top left corner. (B) Hierarchical clustering showing similarities between the profiles for the different groups. The "height" axis indicates the Euclidean distances between groups of profile. A permutation test based on a Kolmogorov-Smirnov statistics indicates a highly-significant difference ($p < 0.0002$) between the profiles for the two main branches of the tree. (C) and (D) Short-term phototropism experiment. Three-day-old etiolated seedlings (4.0 – 5.9 mm hypocotyls) were exposed to blue light (0.1 light grey, 1 intermediate grey or 10 dark grey μmoles $m^{-2}$ $s^{-1}$) for 24 hours. Data are average angles relative to vertical +/- 2X SE (n>95). a: different from the WT grown at 0.1 μmoles $m^{-2}$ $s^{-1}$ $P<0.001$, b: different from the WT grown at 1.0 μmoles $m^{-2}$ $s^{-1}$ $P<0.001$, c: different from the WT grown at 10 μmoles $m^{-2}$ $s^{-1}$ $P<0.001$.

Figure 2

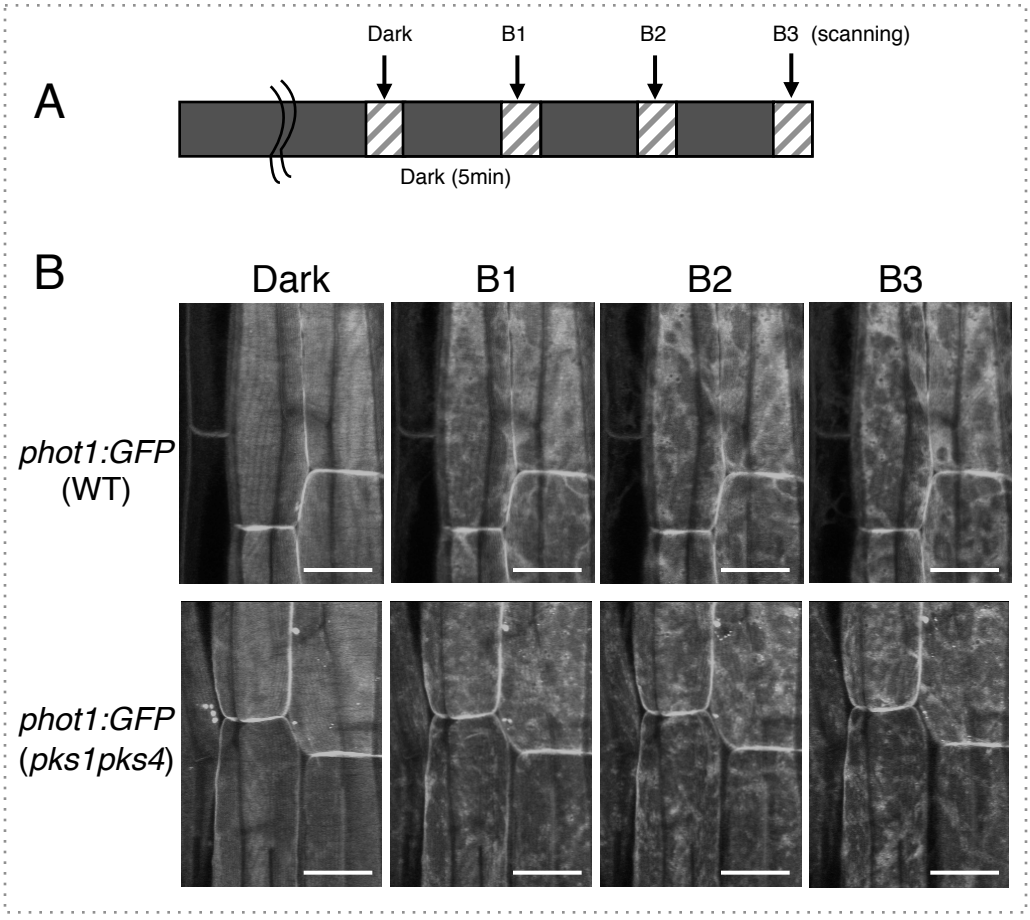



Figure 2. PKS1 and PKS4 are not required for phot1 relocalization under low blue light.
Maximal projection images of phot1:GFP in the *phot1* or *phot1pks1pks4* background. Three-day-old etiolated seedlings (4.0 – 5.9 mm) were scanned (15 section at 1 μm depth intervals) four times with 5min darkness between each scan. Confocal microscopy images were obtained with 488nm laser (1 μmoles $m^{-2}$ $s^{-1}$, 120 seconds scanning time for the 15 sections). Bars = 30 μm.

Figure 3

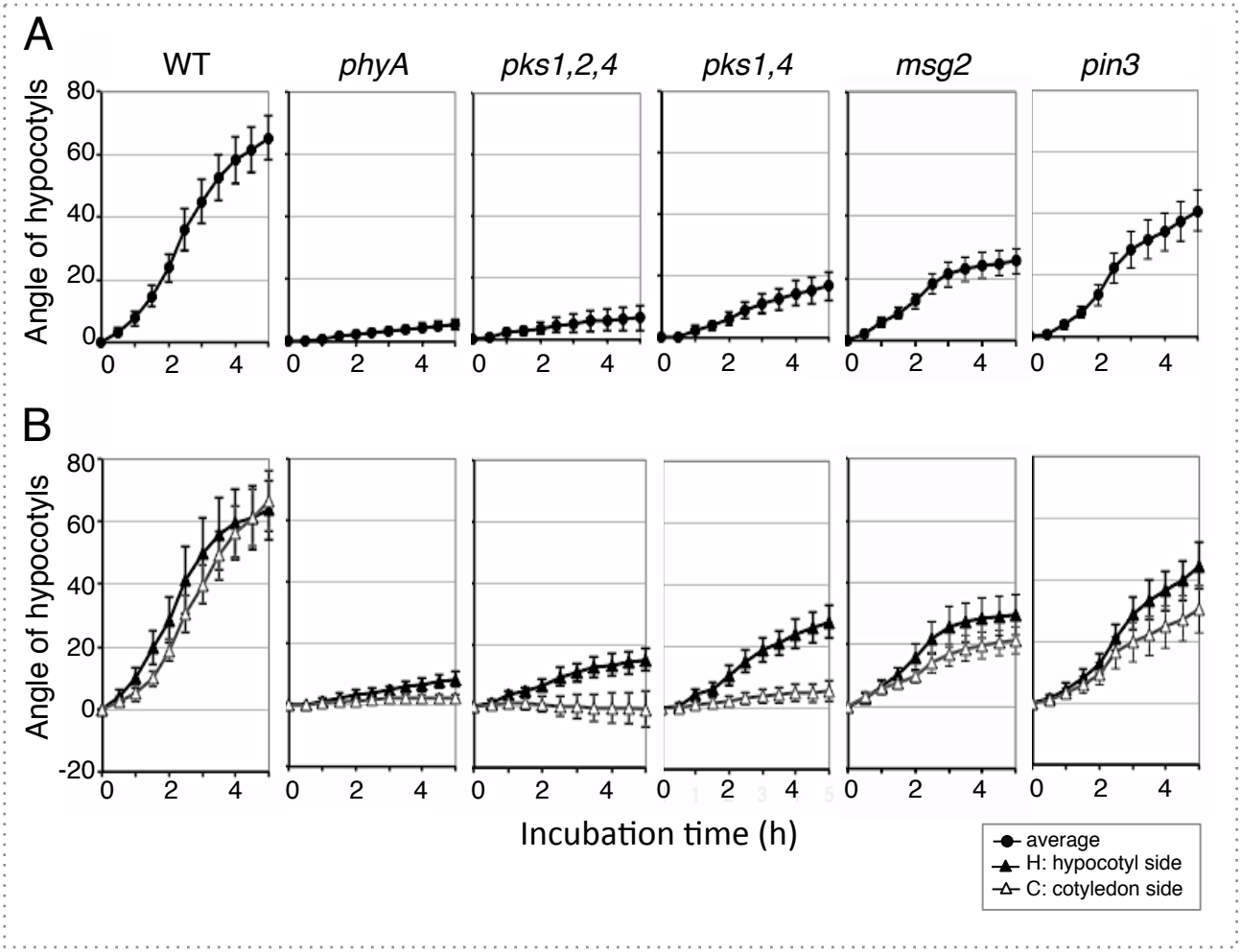



Figure 3. Kinetics of the phototropic response in WT, *phyA, pks1pks4, pks1pks2pks4, msg2* and *pin3.* Dark-grown seedlings (4.0-5.9 mm) were exposed to unilateral blue light (0.5 μmoles $m^{-2}$ $s^{-1}$) for 5 hours. Growth angle of hypocotyls relative to vertical were measured (0° represents vertical growth).

(A) Data are average (full circle) hypocotyl angles from both cotyledon positions (n= 34-40 with 17-20 for each cotyledon position) +/- 2X SE.

(B) Data are average hypocotyl angles for seedlings in H position (hypocotyl towards the light, full triangle) and C position (cotyledon towards the light, open triangle) shown separately. Data are average +/- 2X SE (n= 17-20, each position).

Figure 4

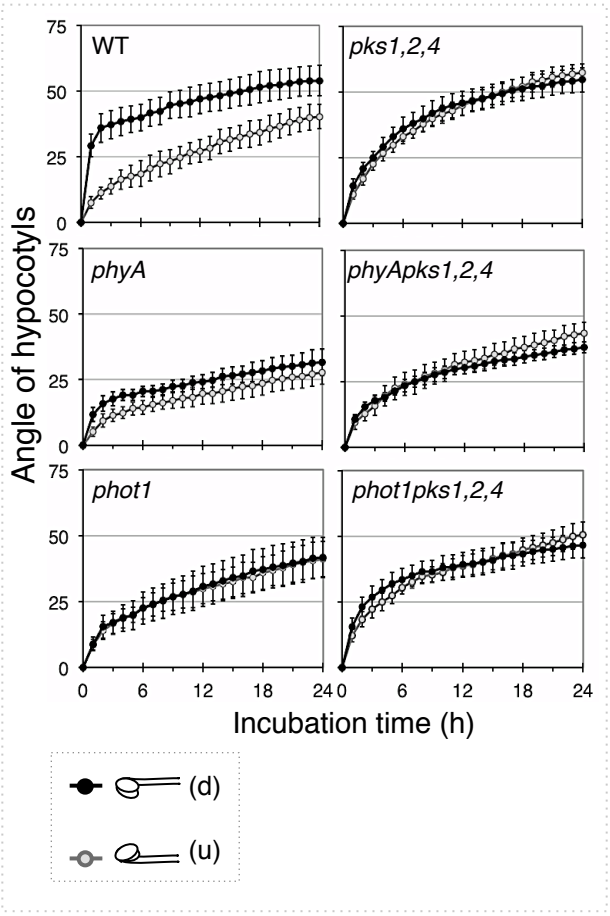



Figure 4. Gravi-reorientation response in WT, photoreceptor mutants and *pks1pks2pks4.*
Three-day-old (4.0 – 5.9 mm) etiolated seedlings of the indicated genotypes were grown on vertical plates. The plates were rotated 90°, and images acquired hourly for 24 hours in darkness. Gravitropic reorientation with different cotyledon positions (d: cotyledons downwards shown as full circles, u: cotyledons upwards shown as open circles). Data are average hypocotyl angles from 20 hypocotyls +/- 2X SE.

Figure 5

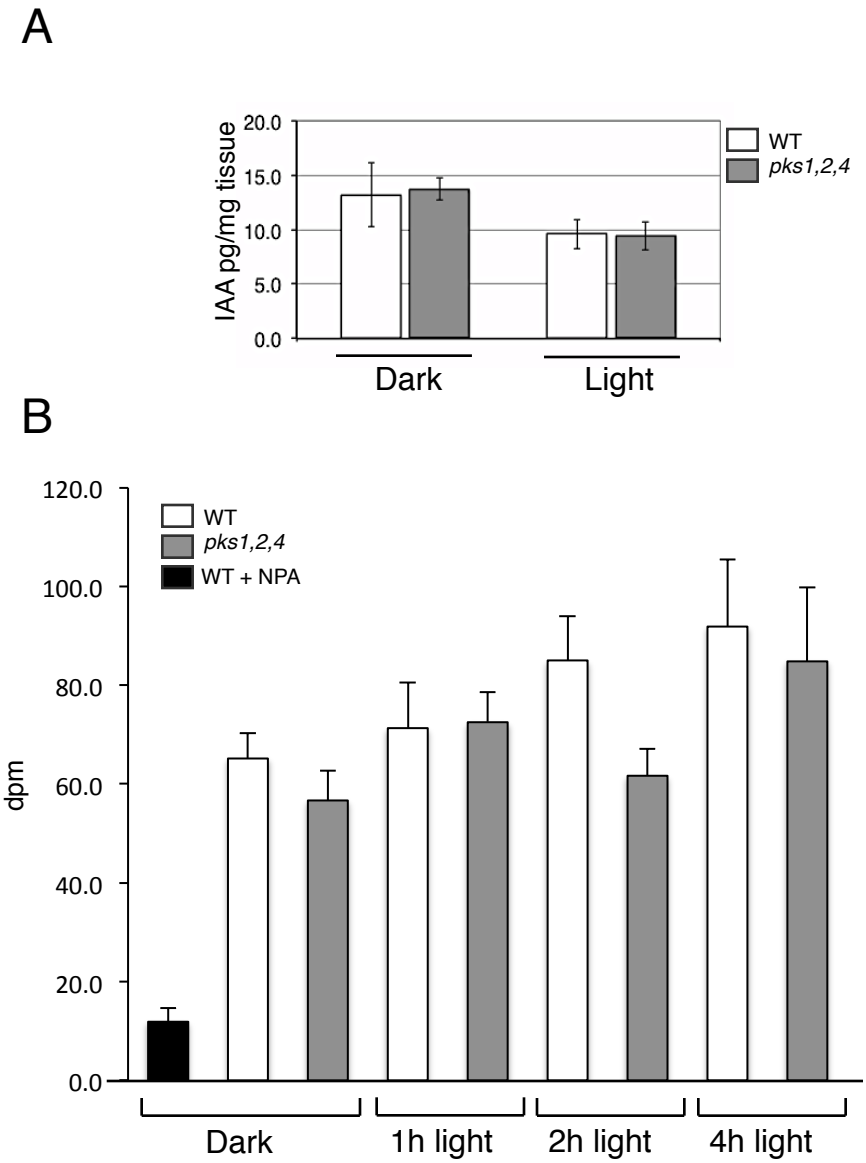



Figure 5. IAA concentration and polar auxin transport in *pks1,2,4* triple mutants.
A: Auxin content of etiolated seedlings (dark) or etiolated seedlings treated for 8 hours with light (20 μmoles $m^{-2}$ $s^{-1}$). Data are average of n=5 +/- 2X SE. B: Polar auxin transport in etiolated Col (WT) and *pks1 pks2 pks4* triple mutants (*pks1, 2, 4*) treated for 0, 1, 2 and 4 hours of light. Data are average of n=8 +/- SE.

Figure 6

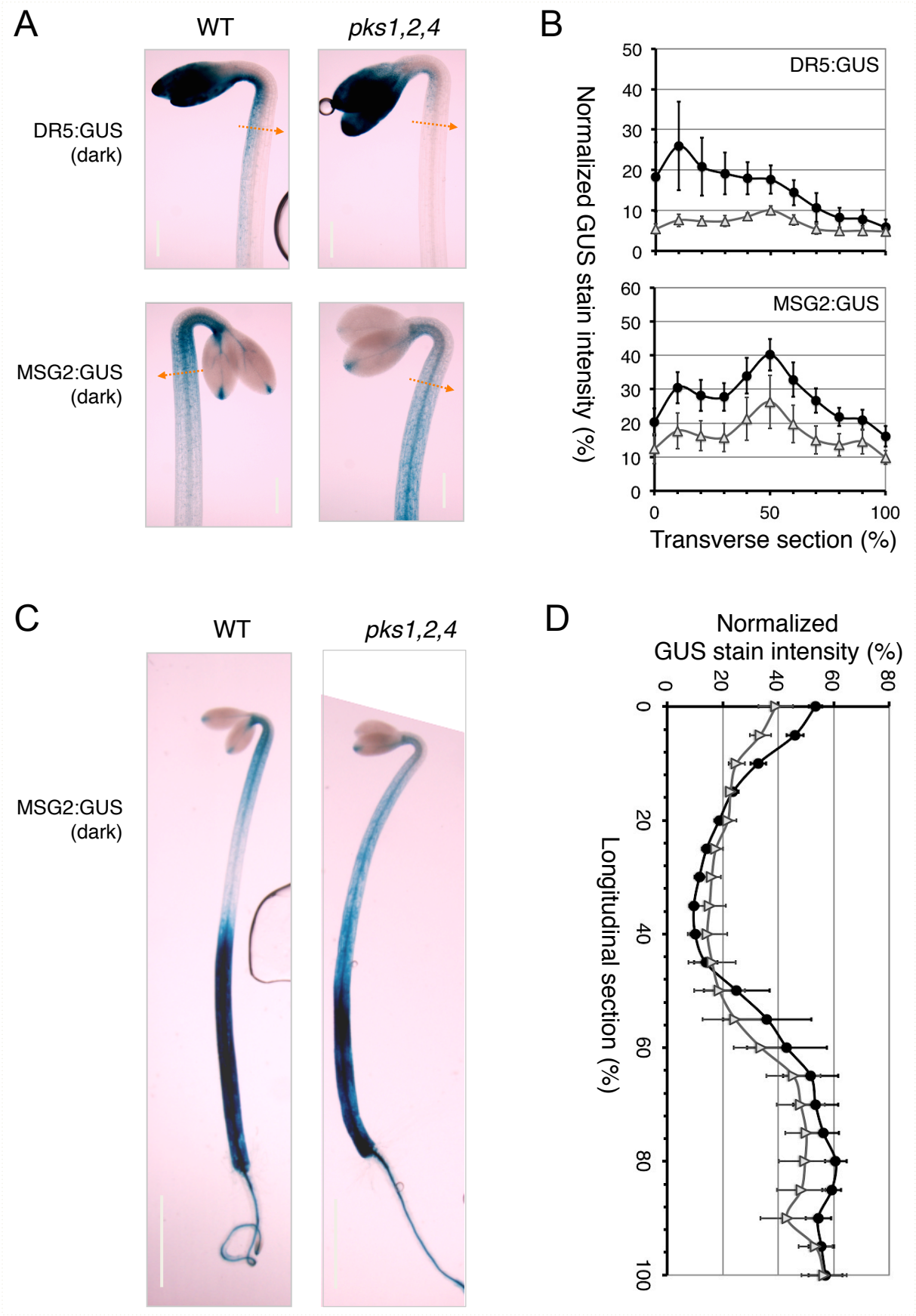
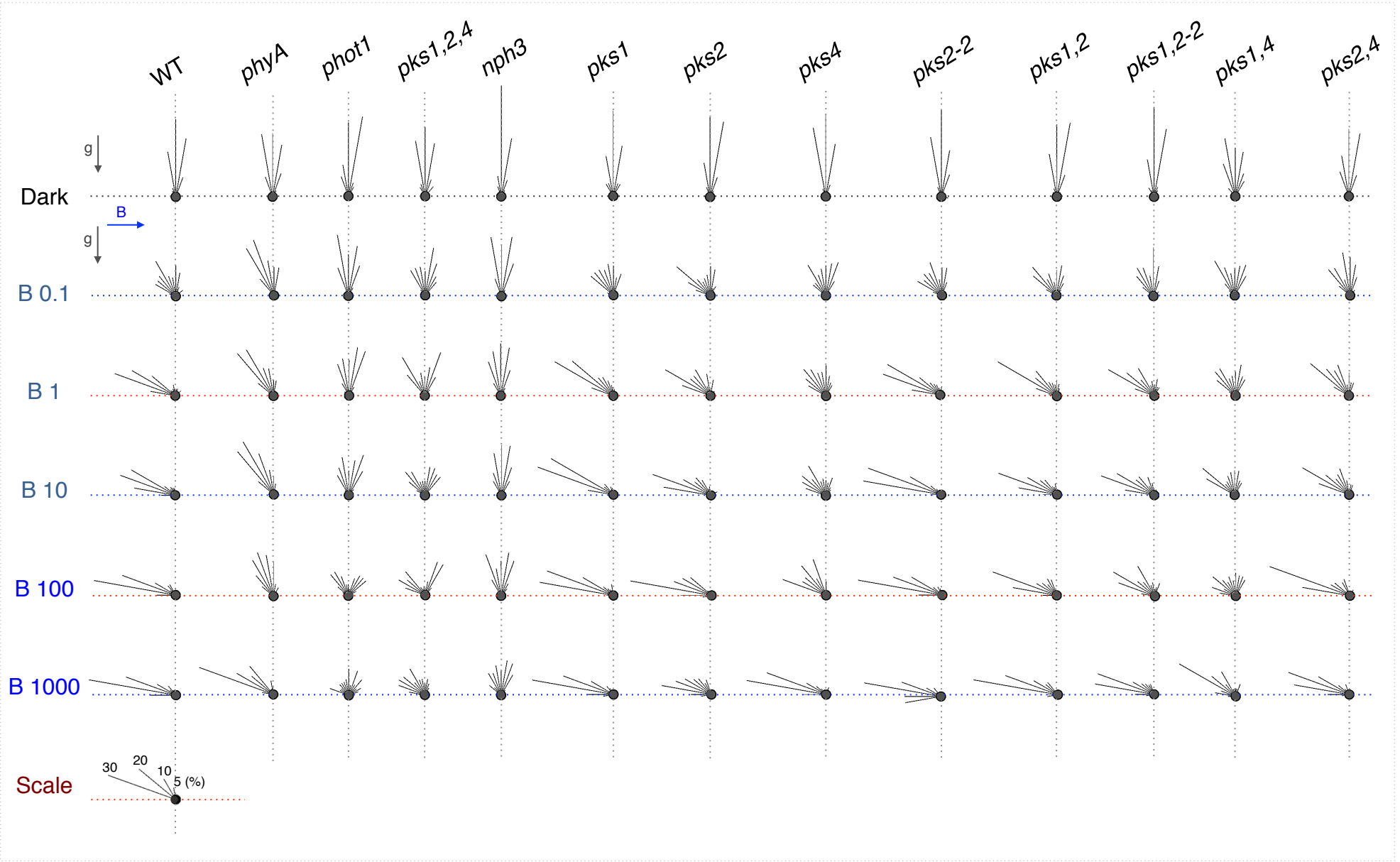

A
WT
pks1,2,4
DR5:GUS
(dark)
MSG2:GUS
(dark)
B
DR5:GUS
MSG2:GUS
Normalized GUS stain intensity (%)
Transverse section (%)
C
WT
pks1,2,4
MSG2:GUS
(dark)
D
Normalized
GUS stain intensity (%)
Longitudinal section (%)

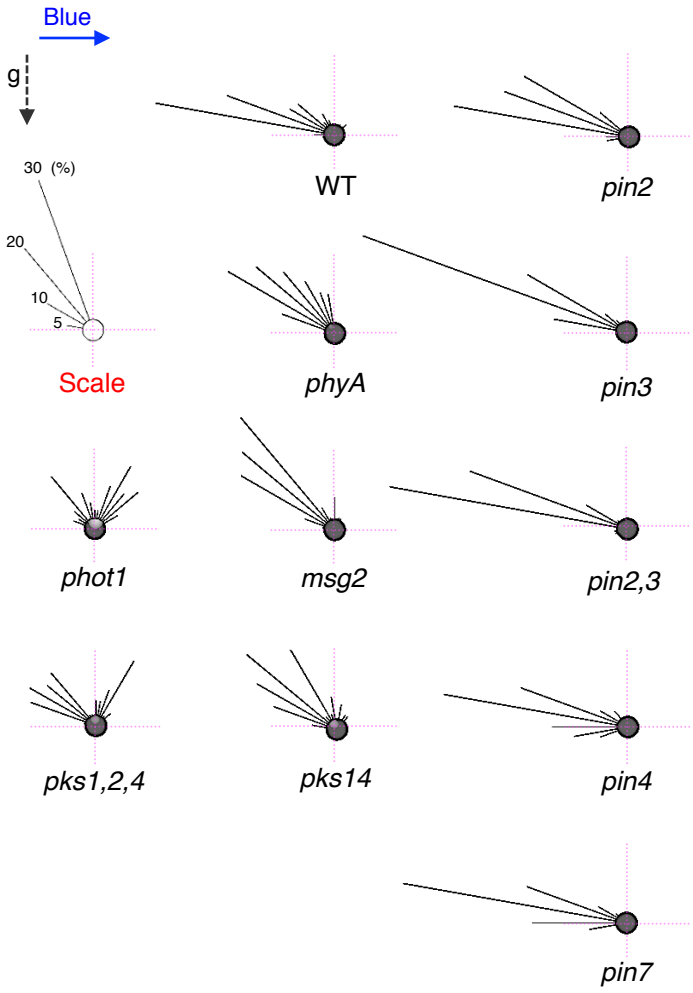
Figure 6. Auxin response is altered in *pks1pks2pks4* mutants. A: Comparison of *DR5:GUS* or *MSG2:GUS* expression between WT and *pks1pks2pks4*. Bar = 250 μm.
B: Normalized GUS stain intensity in elongation zone. Data are average intensity with +/- 2X SE of transverse section from inside of hook to outside (WT: full circle, *pks1,2,4*: open triangle, n=10). The orange arrow indicates the position of the section.
C: Comparison of MSG2:GUS expression between WT and *pks1,2,4*. Bar = 1mm.
D: Normalized GUS stain intensity in hypocotyl. Data are average intensity with +/- 2X SE of longitudinal section from the inside of the hook side from the hook to end of the hypocotyl (WT: full circle, *pks1,2,4*: open triangle, n=10).

Figure S1


WT
phyA
phot1
pks1,2,4
nph3
pks1
pks2
pks4
pks2-2
pks1,2
pks1,2-2
pks1,4
pks2,4
g
Dark
B
g
B 0.1
B 1
B 10
B 100
B 1000
Scale
30
20
10
5 (%)

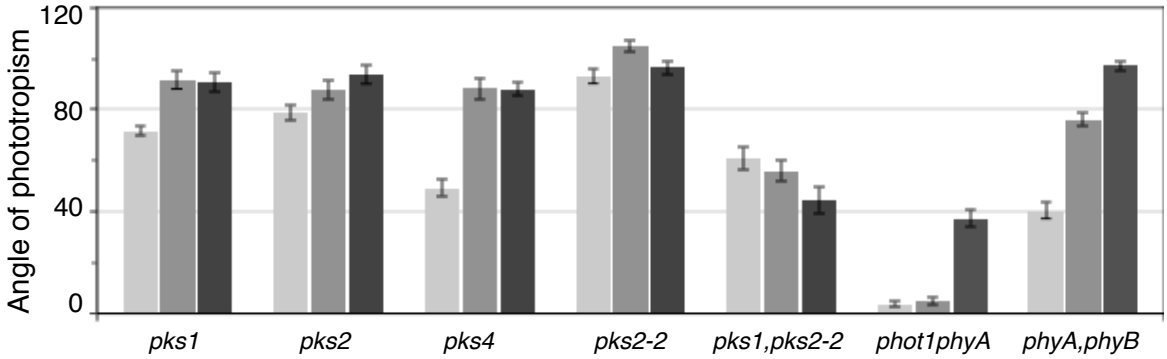
Supplementary Figure S1. Comparison of hypocotyl growth orientation in *phyA, phot1, nph3* and *pks* mutants grown in constant darkness and unilateral blue light (long-term phototropic response). Seedlings were grown on vertical plates for 65 hour with darkness or unilateral blue light (from 0.1 to 1000 n moles $m^{-2}$ $s^{-1}$). Final growth angle relative to vertical was measured (0° represents vertical growth). The direction of growth is shown as circular histograms with 10° angle categories.

Figure S2



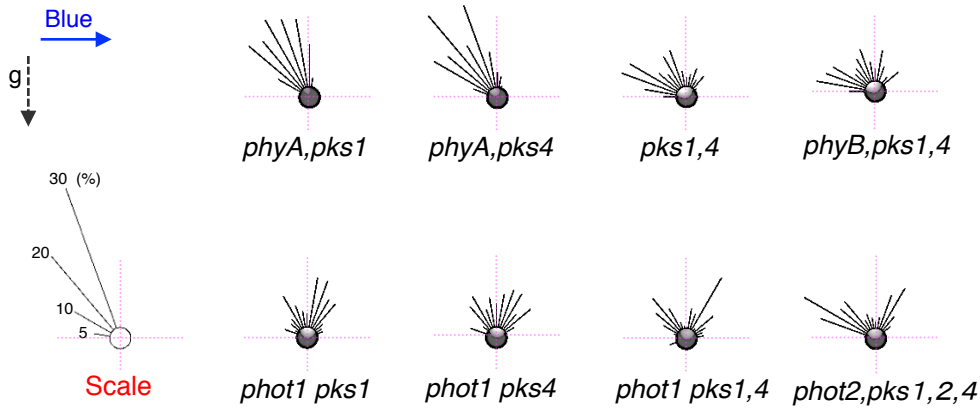

Supplementary Figure S2. Long-term phototropic response of *pks* photoreceptor mutant combinations. Same experiment as presented in Figure 1A for additional genotypes.

Figure S3



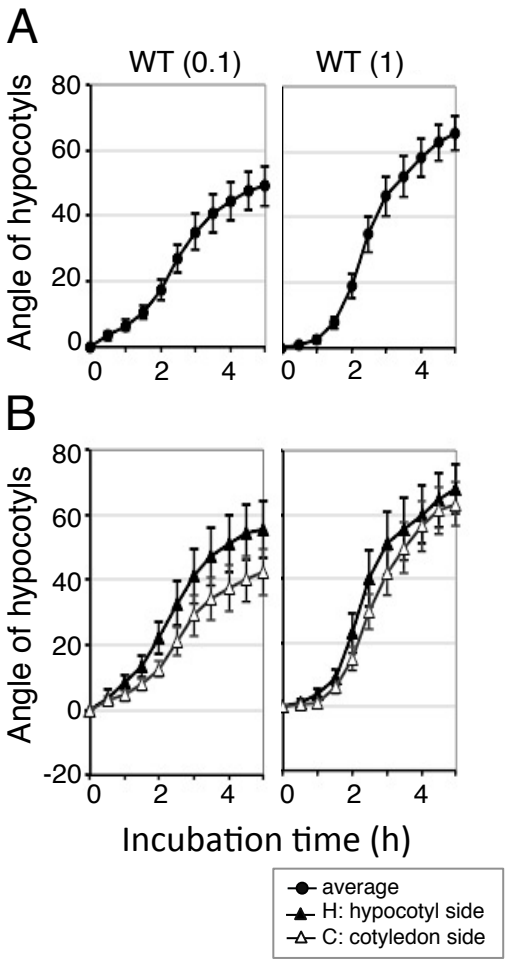

Supplementary Figure S3. Phototropic phenotype of etiolated *pks* mutants treated for 24 hours with different blue light fluence rates. Same experiment as presented in Figure 1C with additional genotypes.

Figure S4




Supplementary Figure S4. Long-term phototropic response of auxin signaling, transport and *pks* mutants. Same experiment as presented in Figure 1A for additional genotypes.

Figure S5

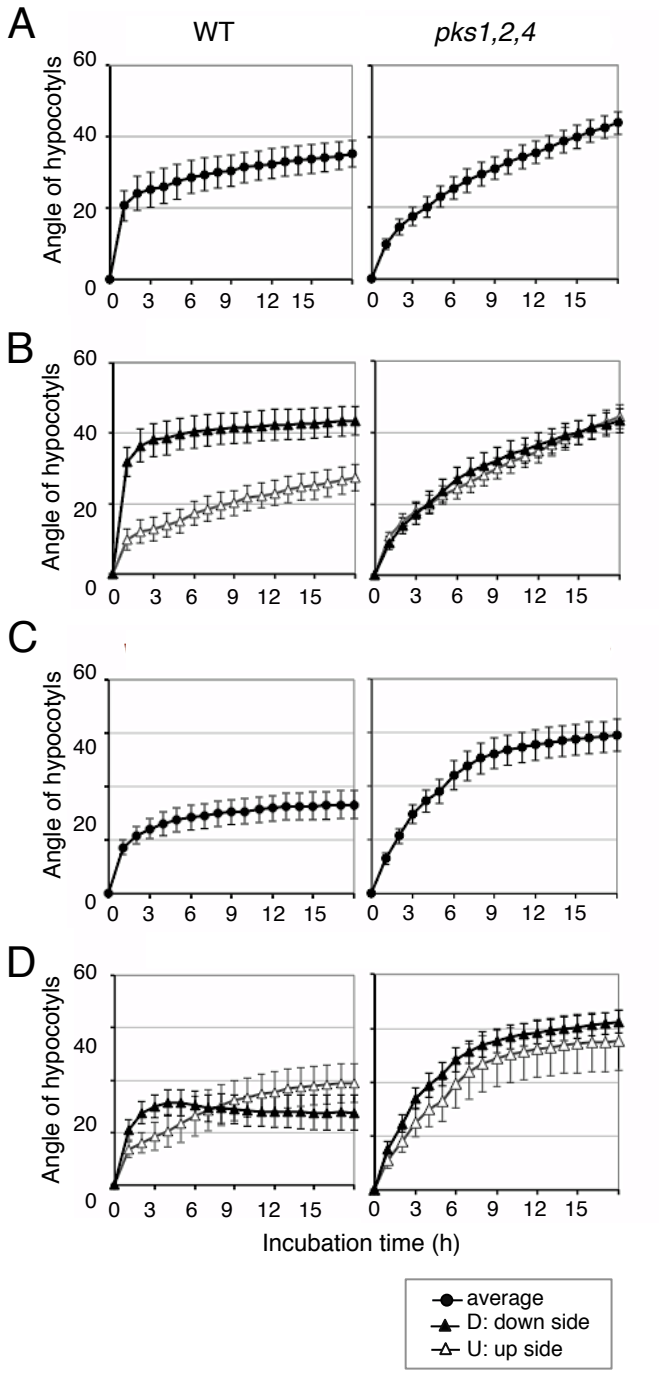



Supplementary Figure S5. Kinetics of the phototropic response in WT under low and high blue light. Same experiment as presented in Figure 3 but with low (0.1 μmoles $m^{-2}$ $s^{-1}$) and intermediate (1 μmoles $m^{-2}$ $s^{-1}$) blue light.

A: Data are average (full circle) hypocotyl angles from both cotyledon position (n= 40, 20 hypocotyls of C and 20 of hypocotyls H) -/+ 2X SE.

B : Phototropism kinetics for position H (full triangle) and C (open triangle) shown separately. Data are average -/+ 2X SE (n= 20, each position)

Figure S6

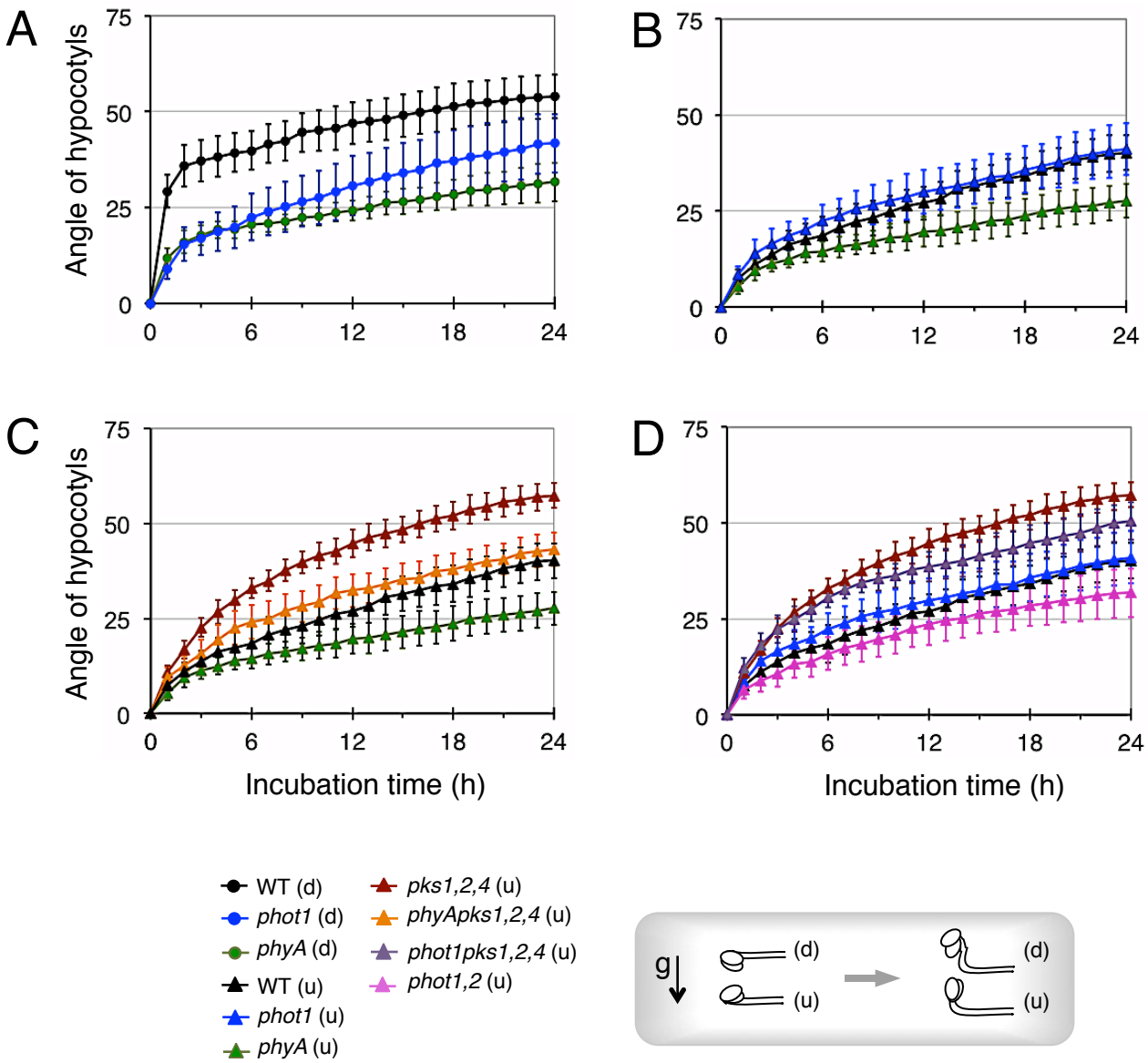



Supplementary Figure S6. Gravi-reorientation responses in WT and *pks1pks2pks4* in darkness and red light. Three-day-old (4.0 – 5.9 mm) etiolated seedlings were grown on vertical plates. The plates were rotated 90°, and images acquired hourly for 18 hours under darkness (A, B) or red light (15 μmoles $m^{-2}$ $s^{-1}$, C, D). Gravitropic reorientation with cotyledon positions (D: cotyledons downwards, U: cotyledons upwards) were measured with ImageJ.

A, C: Data are average (full circle) hypocotyl angles from both cotyledon position (n= 40, 20 hypocotyls of C and 20 of hypocotyls H) +/F 2X SE.

B, D: Data are average hypocotyl angles for position D (full triangle) and U (open triangle) shown separately. Data are average +/F 2X SE (n= 20, each position).

Figure S7




Supplementary Figure S7. Gravi-reorientation responses in WT, *phyA*, *phot1*, *phot1phot2*, *pks1pks2pks4*, *phyApks1pks2pks4* and *phot1pks1pks2pks4* mutants. Data from Figure 4 were plotted differently in order to allow for a better visual comparison of the gravitropic response of the different genotypes.