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Phytochrome Kinase Substrate 4 is phosphorylated by the phototropin 1 photoreceptor

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Phototropism allows plants to redirect their growth towards the light to optimize photosynthesis under reduced light conditions. Phototropin 1 (phot1) is the primary low blue light-sensing receptor triggering phototropism in Arabidopsis. Light-induced autophosphorylation of phot1, an AGC-class protein kinase, constitutes an essential step for phototropism. However, apart from the receptor itself, substrates of phot1 kinase activity are less clearly established. Phototropism is also influenced by the cryptochromes and phytochromes photoreceptors that do not provide directional information but influence the process through incompletely characterized mechanisms. Here, we show that Phytochrome Kinase Substrate 4 (PKS4), a known element of phot1 signalling, is a substrate of phot1 kinase activity *in vitro* that is phosphorylated in a phot1-dependent manner *in vivo*. PKS4 phosphorylation is transient and regulated by a type 2-protein phosphatase. Moreover, phytochromes repress the accumulation of the light-induced phosphorylated form of PKS4 showing a convergence of photoreceptor activity on this signalling element. Our physiological analyses suggest that PKS4 phosphorylation is not essential for phototropism but is part of a negative feedback mechanism.

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Introduction

The survival of living organisms critically depends on accurate perception and responses to external stimuli. Light is a crucial environmental factor that elicits adaptive behaviours in many species, including in plants (Kami *et al*, 2010; Keuskamp *et al*, 2010). Being sessile and photoautotrophic, plants have evolved the ability for plastic growth and development in response to the ever-changing environmental light conditions. They possess sophisticated light-sensing systems allowing them to monitor light quality, quantity, duration and direction (Kami *et al*, 2010; Keuskamp *et al*, 2010). Integration of all these informational cues determines the timing of important developmental transitions (e.g., germination, flowering and senescence) (Mockler *et al*, 2003; Turck *et al*, 2008; Sellaro *et al*, 2009). The light environment also triggers adaptive responses influencing plant morphology in order to optimize light capture and the resulting photosynthetic efficiency (Inoue *et al*, 2008b). In *Arabidopsis thaliana*, five classes of photoreceptors have been identified so far: the recently identified UVR8 perceives Ultraviolet-B (Rizzini *et al*, 2011), phytochromes (phyA to phyE) absorb mainly red and far red light, but also contribute to the perception of blue light together with phototropins (phot1 and phot2), cryptochromes (cry1 and cry2) and members of the zeitlupe (ztl, lkp2 and fkf1) families (Kami *et al*, 2010).

Numerous light-regulated responses such as photoperiodic control of flowering time or de-etiolation, a developmental transition during which the seedling switches from using energy from its seed reserves to photoautotrophic growth are controlled by multiple photoreceptors (Mockler *et al*, 2003; Turck *et al*, 2008; Sellaro *et al*, 2009). This has also been shown for the control of phototropism, a physiological response allowing plants to optimally position their photosynthetic organs towards the light (Whippo and Hangarter, 2003, 2004; Lariguet and Fankhauser, 2004). This photomorphogenic response is important for seedling establishment because carbon availability is the main factor limiting leaf and plant growth in early developmental phases (Pantin *et al*, 2011). While phototropins are responsible for sensing blue light direction, phytochromes and cryptochromes act to modulate the physiological response. For instance, both inhibit gravity-induced vertical orientation of growth providing the seedling with increased flexibility (Lariguet and Fankhauser, 2004; Ohgishi *et al*, 2004). In addition, phytochromes and cryptochromes also affect the responsiveness to the phytohormone auxin, whose differential accumulation between the shaded and lit sides of the hypocotyl is important for the establishment of asymmetric growth (Stowe-Evans *et al*, 2001; Esmon *et al*, 2006; Nagashima *et al*, 2008). Finally, it has been proposed that activation of phytochromes and cryptochromes might

participate more directly to the phototropic response by enhancing phototropin signalling, either by regulating the expression of components of the signalling pathway (Iino, 2006; Lariguet *et al*, 2006; Tsuchida-Mayama *et al*, 2010; Kami *et al*, 2012) or by affecting phot1 subcellular localization (Han *et al*, 2008).

Phototropins possess an amino-terminal light-sensing portion constituted of two Light Oxygen Voltage (LOV) domains (LOV1 and LOV2) and a carboxy-terminal protein kinase domain of the AGC class (Christie, 2007). LOV2 constitutes the major light-sensing domain of phot1, while it has been proposed that LOV1 is involved in modulating photosensitivity (Christie *et al*, 2002; Cho *et al*, 2007). An alpha helix (Jα) undergoing a light-regulated conformational change connects LOV2 to the kinase domain (Harper *et al*, 2003). This conformational change liberates the kinase domain from the inhibitory effect of the amino-terminus of the photoreceptor upon light treatment thus activating phototropin kinase activity (Harper *et al*, 2004; Matsuoka and Tokutomi, 2005; Tokutomi *et al*, 2008). In Arabidopsis, both phot1 and phot2 are phosphorylated upon blue light treatments and several phosphorylation sites have been identified on both light sensors (Inoue *et al*, 2008a, 2011; Sullivan *et al*, 2008). Of particular interest are sites in the activation loop of the kinase domain which when mutated to Ala prevent all tested phototropin response *in vivo* (Inoue *et al*, 2008a, 2011). This suggests that upon blue light perception phototropin autophosphorylation in the activation loop is an essential step for subsequent signalling events. Surprisingly, little is known about the substrates of this kinase activity. The auxin transporter ABCB19 was recently shown to be phosphorylated *in vitro* by phot1 but data demonstrating that this occurs *in planta* are currently not at hand (Christie *et al*, 2011). Phot1-dependent dephosphorylation of the early phototropin signalling component non-phototropic hypocotyl 3 (NPH3) has been reported but the steps leading from phot1 activation to NPH3 dephosphorylation are currently unknown (Pedmale and Liscum, 2007; Tsuchida-Mayama *et al*, 2008).

Members of the Phytochrome Kinase Substrate family (PKS1 to PKS4 in Arabidopsis) have also been proposed to act early in phot1 signalling given that they interact with the photoreceptor and NPH3 at the plasma membrane (Lariguet *et al*, 2006; Boccalandro *et al*, 2008; de Carbonnel *et al*, 2010). Moreover, PKS proteins control phototropin and phytochrome-mediated growth responses, suggesting that they may contribute to photoreceptor co-action during phototropism (Fankhauser *et al*, 1999; Lariguet *et al*, 2003, 2006; Boccalandro *et al*, 2008; Molas and Kiss, 2008; Schepens *et al*, 2008; de Carbonnel *et al*, 2010). In particular, PKS4 has a strong impact on phot1-mediated phototropism and phytochrome-mediated deviation from vertical growth (Lariguet *et al*, 2006; Schepens *et al*, 2008). These findings prompted us to investigate PKS4 protein regulation in response to light treatments eliciting photomorphogenic responses during seedling establishment. We analysed the phosphorylation state of PKS4 given that light-induced phosphorylation events are important early signalling events during photomorphogenesis in particular during phototropin-mediated light responses (Sullivan *et al*, 2008; Inoue *et al*, 2008a, 2011).

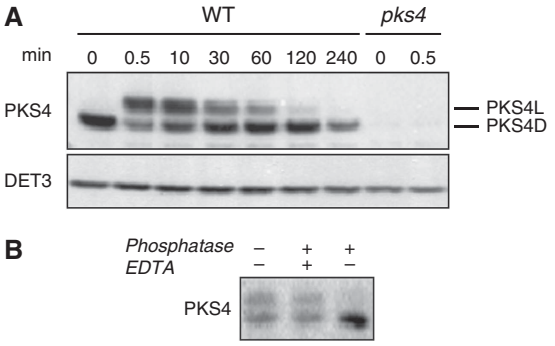

Figure 1 PKS4 is phosphorylated in response to light. (**A**) PKS4 exists as two isoforms in the light. Three-day-old etiolated seedlings (WT) were exposed to constant white light ($140\,\mu mol\,m^{-2}\,s^{-1}$). Total proteins were extracted at the indicated times, separated by SDS–PAGE and transferred onto nitrocellulose membrane. PKS4 accumulation was analysed by immunoblotting using anti-PKS4 antibodies, and DET3 accumulation was used as loading control. Null *pks4* mutant extracts (*pks4*) were used to check anti-PKS4 antibodies specificity. The isoform present in the dark is marked as PKS4D and the one appearing in the light as PKS4L. (**B**) PKS4L formation results from PKS4D phosphorylation. Total proteins extracted from 3-day-old etiolated seedlings illuminated with white light (30 s, $140\,\mu mol\,m^{-2}\,s^{-1}$) were subjected (+) or not (−) to λ-phosphatase treatment (phosphatase) in presence (+) or absence (−) of inhibitor (EDTA).

Results

PKS4 is rapidly and transiently phosphorylated upon light irradiation

Previous studies have shown that *PKS (Phytochrome Kinase Substrate)* gene expression is light regulated and PKS1 protein is phosphorylated (Fankhauser *et al*, 1999; Lariguet *et al*, 2003; Schepens *et al*, 2008). To investigate the regulation of PKS4 at the protein level, we raised an anti-PKS4 antibody. The specificity of the antibody was confirmed by using *pks4* null mutant extracts (Figure 1A). Since PKS4 is required for normal de-etiolation (Lariguet *et al*, 2006; Schepens *et al*, 2008), we analysed PKS4 protein levels in etiolated seedlings transferred to white light. Light treatment resulted in the rapid appearance of an additional slower migrating isoform (Figure 1A). Thus, two co-existing isoforms of PKS4 were detected in the light: a faster migrating form already present in darkness (PKS4D) and a slower migrating form that was only detected upon light treatment (PKS4L).

PKS4L appeared very rapidly upon light treatment, reaching a maximum accumulation within 30 s to 10 min. Accumulation of PKS4L was transient, as it progressively disappeared until being no longer detectable after 4 h in white light (Figure 1A). In general, PKS4D protein levels mirrored those of PKS4L; they decreased upon irradiation and increased following longer irradiation (Figure 1A). These results suggest that a portion of PKS4 is post-translationally modified in a light-dependent manner. Given that another member of the PKS family, PKS1, is phosphorylated upon light treatment, we tested whether generation of PKS4L resulted from protein phosphorylation by subjecting extracts to phosphatase treatments (Fankhauser *et al*, 1999). A reduction of PKS4L levels coupled to an increase in PKS4D accumulation upon phosphatase treatment indicated that PKS4 undergoes light-dependent phosphorylation (Figure 1B).

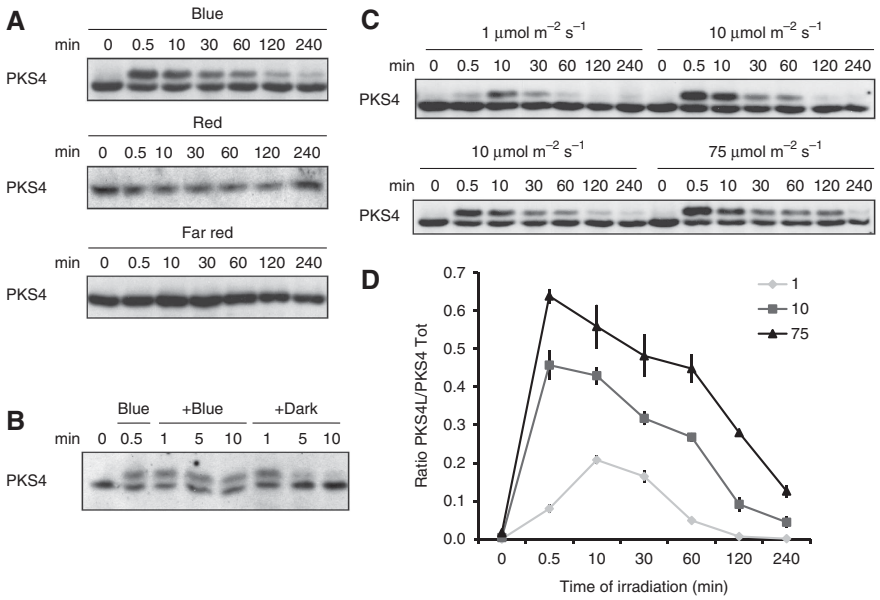

Figure 2 PKS4L accumulates upon blue light perception, specifically. (**A**) Blue light-dependent PKS4 phosphorylation. Three-day-old etiolated seedlings (WT) were exposed to constant blue light ($10\,\mu mol\,m^{-2}\,s^{-1}$), red light ($30\,\mu mol\,m^{-2}\,s^{-1}$) or far-red light ($15\,\mu mol\,m^{-2}\,s^{-1}$). (**B**) PKS4 phosphorylation is reversible. Three-day-old etiolated seedlings were exposed to $10\,\mu mol\,m^{-2}\,s^{-1}$ blue light for 30 s and then kept in blue light or transferred to darkness for the indicated time. (**C**) Fluence rate-dependent PKS4 phosphorylation. Three-day-old etiolated seedlings were exposed to 1, 10 and $75\,\mu mol\,m^{-2}\,s^{-1}$ blue light. Samples treated with $10\,\mu mol\,m^{-2}\,s^{-1}$ blue light are shown on two blots to allow direct comparisons with those treated with 1 and $75\,\mu mol\,m^{-2}\,s^{-1}$ blue light. Proteins were analysed as described in Figure 1. (**D**) Quantification of PKS4L accumulation upon light treatment. Three-day-old etiolated seedlings were exposed to 1, 10 and $75\,\mu mol\,m^{-2}\,s^{-1}$ blue light. Quantifications of PKS4L signal relative to the PKS4D + PKS4L total signal were performed on three biological replicates. Values are means, and error bars represent standard errors.

PKS4 is a phot1 kinase substrate

We next examined the effect of blue, red and far-red light to determine which wavelengths lead to PKS4 phosphorylation. PKS4L was detected only in response to blue light treatment, but not with red or far-red light irradiation (Figure 2A). When seedlings were treated with a pulse of blue light and returned to darkness PKS4L quickly disappeared (Figure 2B). PKS4L accumulation increased in response to higher fluence rates of blue light (Figure 2C). Similarly, the peak of PKS4L accumulation occurred earlier with higher fluence rates (Figure 2C). The influence of the light intensity on PKS4L accumulation was confirmed by quantification of the portion of PKS4 present as PKS4L (PKS4L/PKS4tot) along the time of irradiation (Figure 2D).

To determine which blue light receptor was responsible for these effects, we examined blue light induced PKS4L phosphorylation in phototropin or cryptochrome-deficient mutants. PKS4 phosphorylation was still detected in the cryptochrome double mutant (*cry1cry2*) showing that cryptochromes are dispensable for PKS4L formation (Figure 3A). In contrast, no accumulation of PKS4L was detected in the *phot1phot2* double mutant (Figure 3A). Further genetic analysis revealed that phot1 is the photoreceptor required for PKS4 phosphorylation, as PKS4L accumulation was still detected in *phot2*, but not in *phot1* single mutant (Figure 3A).

Phototropins are composed of an N-terminal photosensory domain that represses the activity of the C-terminal Ser/Thr kinase domain in the dark. Phototropins are activated upon blue light absorption owing to conformational changes that alleviate the repression of kinase activity (Harper *et al*, 2003; Matsuoka and Tokutomi, 2005; Tokutomi *et al*, 2008). A primary step of phot1 signalling is the autophosphorylation of one or two serines (Ser 849 and 851) located within the activation loop of the kinase domain (Inoue *et al*, 2008a). We hypothesized that if light-dependent phosphorylation of PKS4 depends on phot1 kinase activity, then mutations in the kinase domain of phot1 should affect PKS4L formation. We thus examined PKS4L formation in *phot1phot2* double mutants expressing either kinase mutants of phot1 or wild-type phot1 as a control. Phot1 kinase activity is abolished in the kinase-inactivated line ($phot1_{Kin}$) (Christie *et al*, 2002; Cho *et al*, 2007), and diminished in $phot1_{S849AS851A}$ line (Inoue *et al*, 2008a). PKS4L accumulation was no longer detectable in the $phot1_{Kin}$ line and was decreased in the $phot1_{S849AS851A}$ line (Figure 3B). Importantly, altered PKS4L formation in these lines was not due to reduced levels of phot1 expression (Figure 3B). Together, these results indicate that PKS4L accumulation is dependent on phot1 kinase activity.

Both phot1 and PKS4 are strongly expressed in the elongation zone of the hypocotyl (Schepens *et al*, 2008; Wan *et al*, 2008). Moreover similar to PKS1 and PKS2, PKS4 co-immunoprecipitated with phot1-GFP *in vivo* suggesting that phot1 may directly phosphorylate PKS4 (Supplementary Figure S1; Lariguet *et al*, 2006; Boccalandro *et al*, 2008; de Carbonnel *et al*, 2010). We thus tested the ability of phot1 to directly phosphorylate PKS4 *in vitro*. GST-fused recombinant proteins were purified from bacterial extracts. Due to poor solubility and stability of full-length proteins, phot1 and PKS4 were produced as truncated versions (Christie *et al*, 2011; Okajima *et al*, 2011). For PKS4 we tested three different fragments, the N-terminus (amino acids 1–133) and the two C-terminal fragments (amino acids 134–406 and 231–406). The largest PKS4 fragment (amino acids 134–406) was most

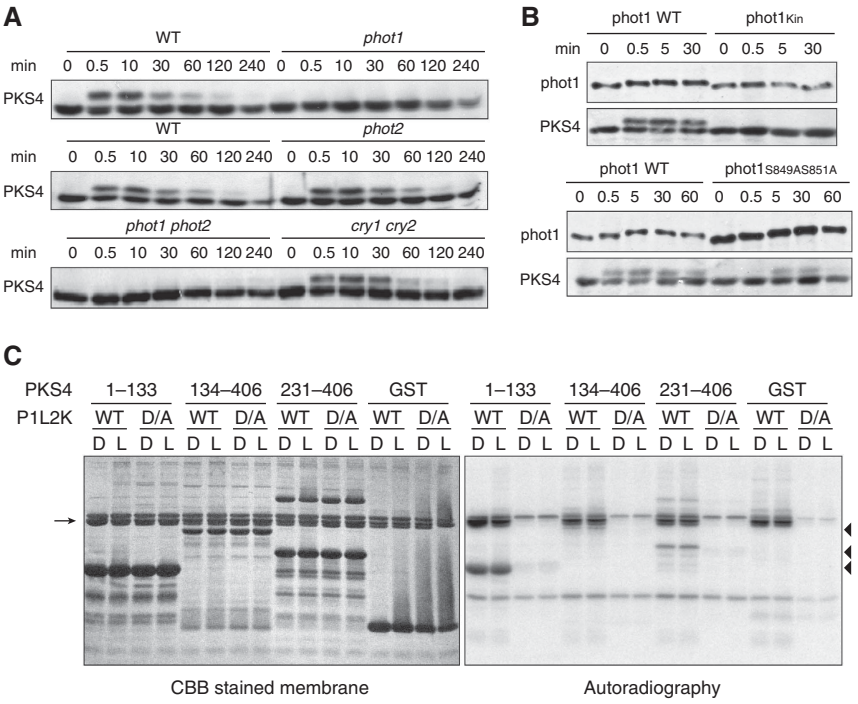

Figure 3 PKS4 is a target of phot1 kinase activity *in vivo* and *in vitro*. (**A**, **B**) PKS4 phosphorylation depends on phot1 kinase activity. Three-day-old etiolated seedlings of Col-0 (WT), *cry1-cry2, phot1-phot2* double mutants, *phot1* and *phot2* single mutants (**A**), or *phot1-phot2* mutant expressing wild-type or mutated versions of phot1 kinase domain (**B**), were exposed to constant blue light ($10\,\mu mol\,m^{-2}\,s^{-1}$ blue light). Total proteins were extracted at the indicated times, separated by SDS–PAGE and transferred onto nitrocellulose membrane. PKS4 and phot1 accumulations were analysed by immunoblotting using anti-PKS4 and anti-phot1 antibodies. (**C**) Phot1 phosphorylates PKS4 fragments *in vitro*. GST-fused PKS4 polypeptides (PKS4 amino acids 1–133, 134–406 or 231–406; position marked by dark triangles) or GST were used as substrates for *in-vitro* phosphorylation assay in presence of GST-fused LOV2-Kinase domain polypeptides of phot1 either wild-type version or D788A kinase inactive mutated version (P1L2K WT and D/A, position marked by arrow). The reaction was performed under blue light (L) or mock irradiation (D). Proteins were separated on 15% acrylamide gel and transferred onto PVDF membrane. Left panel corresponds to Coomassie blue staining of the membrane. Right panel corresponds to autoradiography.

difficult to express and purify and we were unable to produce fractions as concentrated as for the other two. Phosphorylation assays were performed either in darkness or in presence of light. Even though no light regulation was observed, phosphorylation of the N-terminal PKS4 fragment and the most C-terminal PKS4 fragment were clearly detected in presence of active phot1 kinase but not in presence of a kinase inactivated version (Figure 3C). Phosphorylation of the third PKS4 fragment (amino acids 134–406) is detected as a faint band just below phot1 (P1L2K). The apparently weaker phosphorylation of this fragment of PKS4 might simply result from the lower concentration of substrate in these reactions (see Coomassie stained membrane) (Figure 3C). As observed previously autophosphorylation of phot1 also occurred independently of the light treatment (Christie *et al*, 2011; Okajima *et al*, 2011). We conclude that PKS4 is a substrate of phot1 kinase *in vitro*.

PKS4L accumulation is repressed by phytochromes under low blue light intensity

Phytochromes are red/far-red photoreceptors, but are also known to mediate blue light responses (Kami *et al*, 2010). We thus analysed their involvement in PKS4L accumulation. PKS4L still accumulated in the quintuple phytochrome mutant (Figure 4). The PKS4L/PKS4tot ratio was not strikingly affected by the absence of phytochrome activity in high blue light condition where this mutant still shows phototropism (Figure 4A; Strasser *et al*, 2010). However, PKS4L accumulation was strongly enhanced in the quintuple phytochrome mutant when seedlings were irradiated with low fluence rates of blue light where phytochromes are known to promote phototropism (Figure 4B; Lariguet *et al*, 2006; Tsuchida-Mayama *et al*, 2010; Kami *et al*, 2012). This result shows that phytochromes negatively regulate PKS4L accumulation in low blue light conditions.

A striking example of concerted action between phot1 and the phytochromes is that phot1-mediated phototropism is enhanced by phytochrome photoactivation (Liscum and Briggs, 1996; Parks *et al*, 1996). Irradiation of seedlings with a pulse of red light prior to blue light irradiation accelerates the phototropic response. In the wild type, red light pre-treatment slightly but significantly decreased the PKS4L/PKS4tot ratio. However, this effect was not observed in the quintuple phytochrome mutant (Figure 4B). This result confirms that phytochromes repress the accumulation of PKS4L and shows that PKS4D to PKS4L inter-conversion is controlled by the antagonistic action of two photoreceptors: phot1 promotes PKS4 phosphorylation while phytochromes repress the accumulation of the phosphorylated form.

PKS4L formation occurs upstream of auxin signalling/redistribution

In etiolated seedlings, phosphorylation of PKS4 depends on phot1 and is modulated by the phytochromes (Figures 3 and

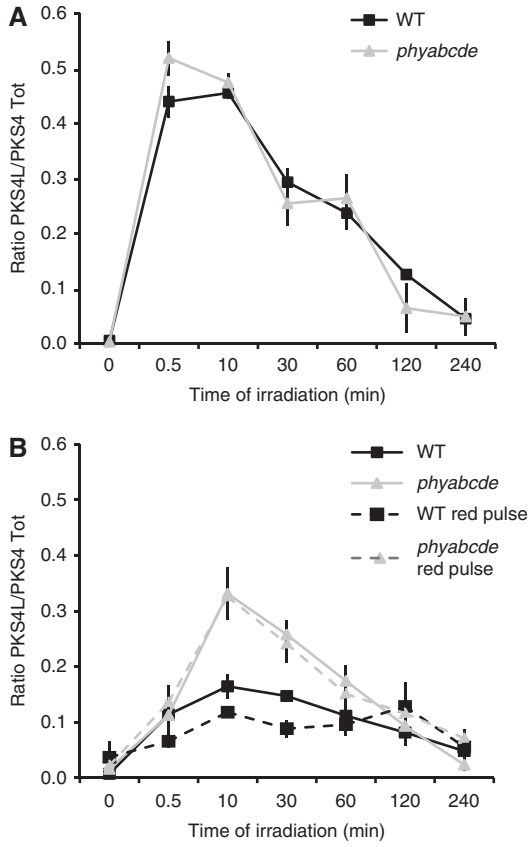

Figure 4 Phytochromes modulate PKS4 phosphorylation. (**A**) Phytochromes have a very weak effect on PKS4L accumulation in high blue light intensity. Three-day-old etiolated seedlings of wild-type (WT) and the quintuple phytochrome mutant (*phyabcde*) were irradiated with $10\,\mu mol\,m^{-2}\,s^{-1}$ blue light. Quantifications of PKS4L signal relative to the PKS4D + PKS4L total signal were performed on three biological replicates. Values are means, and error bars represent standard errors. (**B**) Phytochromes decrease PKS4L accumulation in low blue light intensity and upon red light pre-irradiation. Three-day-old etiolated seedlings of WT and the quintuple phytochrome mutant (*phyabcde*) were irradiated with $30\,\mu mol\,m^{-2}\,s^{-1}$ red light for 1 min or mock irradiated, kept in darkness for 1 h, then irradiated with $1\,\mu mol\,m^{-2}\,s^{-1}$ blue light for the indicated time. Quantifications of PKS4L signal relative to the PKS4D + PKS4L total signal were performed on four biological replicates. Values are means, and error bars represent standard errors.

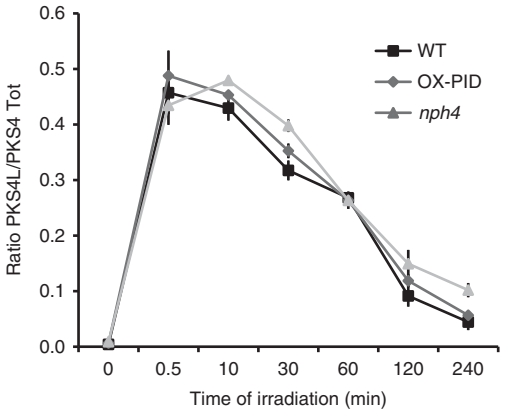

Figure 5 PKS4 phosphorylation occurs upstream of auxin signalling. Three-day-old etiolated seedlings (WT, OX-PID, *nph4*) were irradiated with $10\,\mu mol\,m^{-2}\,s^{-1}$ blue light. Quantifications of PKS4L signal relative to the PKS4D + PKS4L total signal were performed on three biological replicates. Values are means, and error bars represent standard errors.

4). Following phototropin activation, a gradient of auxin forms in the hypocotyl that precedes asymmetric growth of the embryonic stem (Esmon *et al*, 2006). PKS4 phosphorylation is very rapid and phot1 dependent (Figure 3) and we wished to determine whether PKS4L appearance is affected by auxin transport or signalling. Tropic growth and auxin transport are altered in seedlings overexpressing PINOID (OX-PID; Ding *et al*, 2011), but the appearance of PKS4L upon light stimulation was normal in this genetic background (Figure 5). Similarly, PKS4 phosphorylation was not affected in the phototropism-deficient auxin-signalling mutant *nph4* (Figure 5; Harper *et al*, 2000). Collectively, these data indicate that the phot1-dependent PKS4 phosphorylation is an early response occurring upstream of auxin redistribution and the NPH4-mediated transcriptional response.

RCN1-dependent PKS4 dephosphorylation

The conversion of PKS4D to PKS4L is transient and reversible as a decrease in the PKS4L isoform is paralleled by an increase in PKS4D (Figures 1A and 2B). Given that PKS4L is a phosphorylated isoform its disappearance upon prolonged blue light treatment could be due to dephosphorylation. To test this hypothesis, we treated etiolated seedlings with the phosphatase inhibitor cantharidin (CN) 1 h before blue light irradiation (Li *et al*, 1993). We observed that PKS4L accumulation was higher when the seedlings were subjected to CN treatment (Figure 6A), supporting the hypothesis that the disappearance of PKS4L involves phosphatase activity. In the dark, PKS4 exists as a single isoform. The absence of PKS4L in the dark could result either from the absence of phosphorylation or from a high phosphatase activity that counteracts phosphorylation. The fact that PKS4L was not detected in darkness in the presence of CN excluded the latter possibility and confirms that PKS4L formation is due to light-induced phosphorylation that correlates with the phot1 activity.

CN is a selective inhibitor of PP1 and PP2A phosphatases, but has a higher affinity for PP2A than PP1 (Li *et al*, 1993). The phot1-interacting protein NPH3 is reported to be a PP1 target (Pedmale and Liscum, 2007). NPH3 is dephosphorylated in response to blue light in a process that is prevented in the presence of 50 μM CN (Pedmale and Liscum, 2007). Our studies indicate that PKS4 phosphorylation has a higher sensitivity to CN than NPH3. Indeed, 10 μM CN was sufficient to increase strongly PKS4L accumulation but had no obvious effect on NPH3 dephosphorylation (Figure 6A). The higher sensitivity of PKS4 to CN compared to NPH3 suggests that PKS4 dephosphorylation could be mediated by PP2A activity rather than PP1. To test this hypothesis, we examined PKS4L/PKS4tot ratio in a mutant affected for PP2A activity. PP2As are heterotrimeric Ser/Thr-specific protein phosphatases, composed of one catalytic C subunit and two regulatory subunits, A and B. RCN1 has been identified as one of the three regulatory subunits A in Arabidopsis. Loss of RCN1 function strongly impairs global PP2A activity in Arabidopsis (Deruere

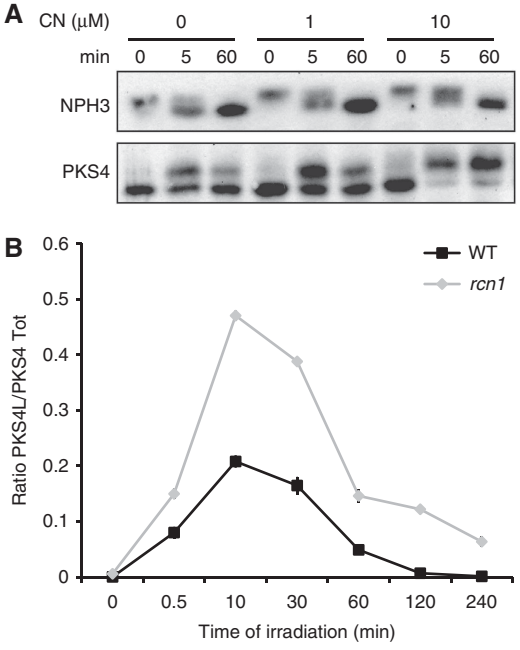

Figure 6 PP2A activity modulates PKS4 phosphorylation. (**A**) PKS4L accumulation is enhanced upon phosphatase inhibitor treatment (CN = cantharidin). Three-day-old etiolated seedlings were transferred onto medium containing 0, 1 and 10 μM CN 1 h before light exposure. Proteins were extracted after 0, 5 and 60 min of 15 μmol m^{-2} s^{-1} blue light irradiation and analysed as described in Figure 2. (**B**) PKS4L accumulation is enhanced in *rcn1* mutant. Three-day-old etiolated seedlings (WT and *rcn1*) were irradiated with 1 μmol m^{-2} s^{-1} blue light for the indicated time. Quantifications of PKS4L signal relative to the PKS4D + PKS4L total signal were performed on three biological replicates. Values are means, and error bars represent standard errors.

et al, 1999). Consistent with our pharmacological studies, the PKS4L/PKS4D ratio was higher in *rcn1* mutant as compared to the wild type at each time point of our analysis (Figure 6B). This result indicates that PKS4D to PKS4L inter-conversion is regulated by PP2A activity.

Phosphorylation of PKS4 negatively regulates phototropism

Upon blue light perception, rapid inhibition of hypocotyl growth precedes the onset of curvature. This process is rapid, phot1 and fluence rate dependent, reminiscent of the formation of PKS4L (Figures 2 and 3; Folta and Spalding, 2001). Given the similarities between these two events, we examined the role of PKS4 in the rapid inhibition of hypocotyl elongation by blue light. Our results confirmed the fluence-rate dependent feature of this response and the essential role of phot1, however, the *pks4* mutant displayed a response similar to the wild type (Figure 7).

Since PKS4 plays a positive role in phototropism (Lariguet *et al*, 2006), we hypothesized that PKS4 phosphorylation may modulate the phototropic response. Two experiments suggested that phosphorylation of PKS4 was not required for phototropism. First in *phot1phot2* plants expressing a truncated version of phot1 (L2K) that is sufficient to enable phototropism (Sullivan *et al*, 2008) we saw no light-induced phosphorylation of PKS4 suggesting that the appearance of PKS4L is not a prerequisite for phototropism. In addition, robust appearance of PKS4L was most efficient at fluence

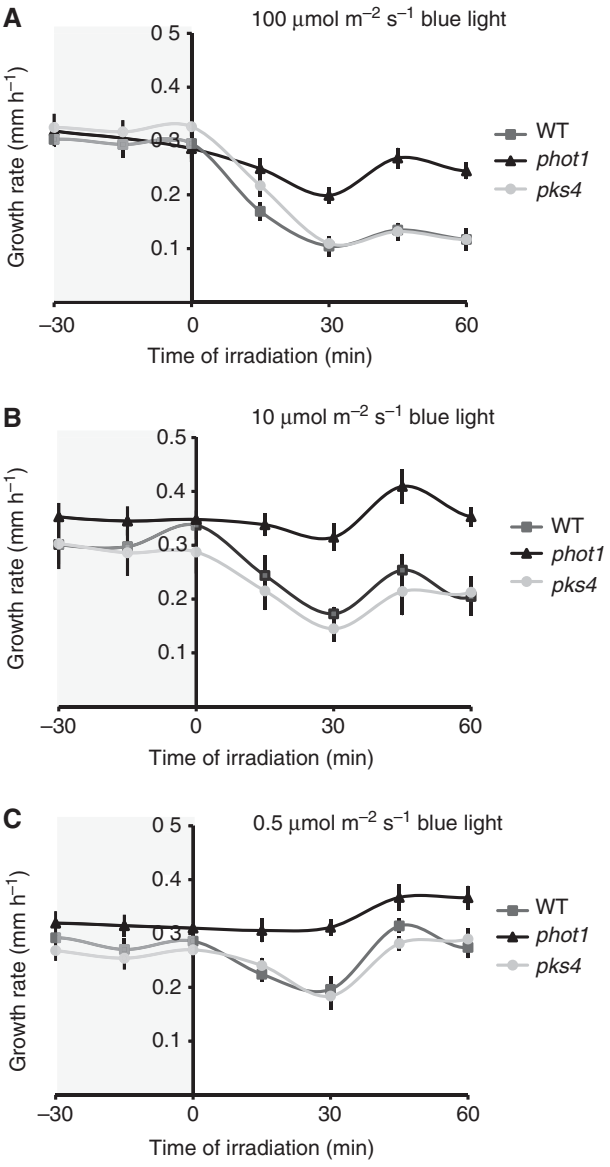

Figure 7 PKS4 is not essential for phot1-dependent hypocotyl growth inhibition. Two-day-old etiolated seedlings (WT, *pks4*, *phot1*) were irradiated with 100 μmol m^{-2} s^{-1} (**A**), 10 μmol m^{-2} s^{-1} (**B**) or 0.5 μmol m^{-2} s^{-1} (**C**) blue light from above. Data represent means of hypocotyl growth rates in darkness (−30 to 0 min) or upon light treatment (0–60 min). Bars indicate two standard errors ($n > 20$).

rates that are much higher than those required to induce phototropism (Figure 2). We thus envisaged the possibility that PKS4 phosphorylation may rather inhibit phototropism, which is known to occur in response to high fluences of light in particular when phototropism is triggered by pulses of light (Konjevic *et al*, 1989; Janoudi and Poff, 1991).

The relationship between the total fluence of light and the phototropic response is complex with an early phase where the two are proportional followed by a phase where increasing blue light fluence inhibits phototropism and finally time-dependent phototropism that is most often studied (Konjevic *et al*, 1989; Iino, 2006). The mechanisms leading to an inhibition of phototropism in response to increasing fluences of blue light are unknown. In order to test whether

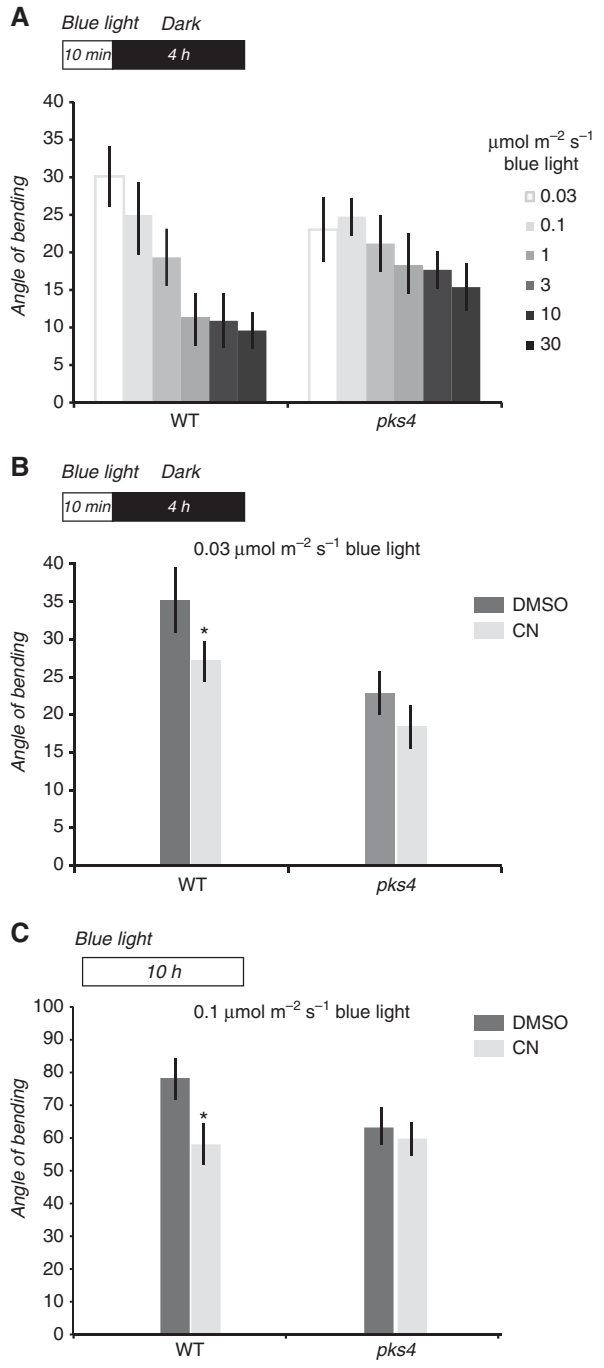

Figure 8 PKS4L accumulation negatively regulates phototropism. (**A**) Enhanced PKS4L accumulation by increasing blue light intensity inhibits pulse-induced phototropism. Three-day-old etiolated seedlings (WT and *pks4*) were irradiated for 10 min with unilateral blue light (0.03, 0.1, 1, 3, 10 or 30 $\mu mol\,m^{-2}\,s^{-1}$) returned to darkness for 4 h before measuring the phototropic curvature. (**B**, **C**) Enhanced PKS4L accumulation by cantharidin (CN) treatment inhibits phototropism. Three-day-old etiolated seedlings (WT and *pks4*) were transferred onto agar plates containing 10 µM cantharidin (CN) or mock treated (DMSO) and returned to darkness for 1 h before the light treatment. (**B**) Seedlings were treated for 10 min with 0.03 $\mu mol\,m^{-2}\,s^{-1}$ blue light, returned to darkness for 4 h before measuring phototropic curvature. (**C**) Seedlings were treated for 4 h with 0.1 $\mu mol\,m^{-2}\,s^{-1}$ unilateral blue light before measuring phototropic curvature. Data are means ± two standard errors ($n > 20$). *Indicates significant differences between means of CN versus DMSO (*P*-value < 0.05).

PKS4 and its phosphorylation were involved in this process wild-type and *pks4* seedlings were irradiated with unilateral blue light for 10 min with variable fluence rates (leading to different PKS4L/PKS4tot ratios; Figure 2) and phototropism measured after returning them into darkness for 4 h (Figure 8A). Curvature of wild-type seedlings decreased by increasing the fluence rate of irradiation (significant difference with *P*-value < 0.05 between 0.03 and fluence rate higher than 0.1 $\mu mol\,m^{-2}\,s^{-1}$). *pks4* mutant displayed reduced phototropism at the lowest fluence rate tested (0.03 $\mu mol\,m^{-2}\,s^{-1}$), confirming the positive role of PKS4 for phototropism reported previously (Lariguet *et al*, 2006). Increasing the fluence rate decreased curvature of *pks4* mutant. However, *pks4* was less sensitive than the wild type to the light intensity effect (significant difference with *P*-value < 0.05 between 0.03 and fluence rate higher than 3 $\mu mol\,m^{-2}\,s^{-1}$). Consequently, *pks4* showed stronger phototropism than wild type for the highest fluence rates tested. Since increasing the fluence rates increased PKS4L accumulation (Figure 2D) while inhibiting phototropism (Figure 8A), we hypothesized that PKS4L may act as a negative regulator of phototropism.

To further test whether PKS4L inhibits phototropism, we treated seedlings with CN leading to increased PKS4L formation in low light (Figure 6) and subjected those seedlings to unilateral blue light to test their phototropic response (Figure 8B). Treatment with CN impaired hypocotyl curvature in wild-type seedlings (Figure 8B). However, CN treatment had no significant effect on the curvature response of *pks4* seedlings. This suggests that CN effect on WT phototropic responsiveness is at least partially due to enhanced accumulation of PKS4L. We also examined the effect of CN on phototropism under prolonged light irradiation (Figure 8C). In this condition, phototropic responsiveness was also decreased in WT but not in *pks4*. This result again indicates that stabilization of PKS4L during the time of irradiation correlates with reduced phototropism. Altogether our physiological results suggest that PKS4 has a dual role in phototropism: PKS4D is a positive regulator whereas PKS4L is a negative regulator of phototropism (Figure 8).

Discussion

PKS4 is a substrate of phot1 signalling

In this study, we show that PKS4 undergoes blue light-dependent phosphorylation that is mediated by phot1 kinase activity. Transphosphorylation catalysed by phot1 was previously demonstrated *in vitro* using the N-terminal region of phot1 or the auxin transporter ABCB19 as substrates (Christie *et al*, 2011; Okajima *et al*, 2011). Here, we present evidence that PKS4 is a target of phot1 kinase activity both *in vivo* and *in vitro* (Figure 3; Supplementary Figure S1). Phot1 LOV2-linker-kinase (P1L2K) phosphorylates both the N-terminal (amino acids 1–233) and the C-terminal (amino acids 231–406) regions of PKS4 indicating the existence of multiple phosphorylation sites. Light regulation of phot1-dependent PKS4 phosphorylation was clearly observed *in vivo* but not *in vitro* (Figure 3). The P1L2K phosphorylates an N-terminal portion of phot1 in a light-dependent manner, indicating that P1L2K can act as a light-regulated kinase (Okajima *et al*, 2011). However, PKS4 polypeptides did not show marked light-inducible phosphorylation. Several reasons may

explain the absence of light regulation *in vitro*. First, autophosphorylation of P1L2K itself is not light regulated (Okajima *et al*, 2011; Figure 3). A longer fragment of the phot1 polypeptide, for example, including the N-terminal flanking region of LOV2 that is necessary for Chlamydomonas phototropin light regulation, may be necessary for light regulation of PKS4 phosphorylation *in vitro* (Aihara *et al*, 2012). In addition, full-length PKS4 may present a particular conformation potentially lost in the fragments, which allows its light-regulated phosphorylation. Finally, in contrast to the *in-vivo* situation where both the kinase and the substrate are associated with the plasma membrane (Supplementary Figure S1; Lariguet *et al*, 2006; Wan *et al*, 2008), these assays are performed with soluble components which may compromise light regulation of the reaction.

In planta PKS4 phosphorylation occurs very early following phot1 activation and does not depend upon auxin redistribution that precedes phototropism (Figures 3 and 5). PKS4L accumulation and autophosphorylation of phot1 share common features: they are both fluence rate dependent and reversible (Salomon and Knieb, 2003; Inoue *et al*, 2008a; Sullivan *et al*, 2008; Figure 2). Moreover, phot1-dependent PKS4 phosphorylation and phot1 autophosphorylation within the activation loop (at Ser 851) exhibit similar kinetics of dephosphorylation upon treatment with a blue light pulse (Inoue *et al*, 2008a; Figure 3B). The level of PKS4L is also influenced by the degree of phot1 kinase activity (Figure 3C). While PKS4L is absent in seedlings expressing a kinase-inactive version of phot1, expression of phot1$_{S849AS851A}$ restores light-dependent PKS4 phosphorylation albeit at a lower levels as compared to the wild type (Figure 3C). The observation that PKS4 phosphorylation still occurs in this line can be explained by the fact that phosphorylation within the activation loop of kinases typically enhances their activity, but is not always necessary for substrate recognition (Adams, 2003). Together with previous studies, our data reinforce the notion that early steps in phot1 signalling primarily involve changes in protein phosphorylation status (Ueno *et al*, 2005; Pedmale and Liscum, 2007; Sullivan *et al*, 2009).

PKS4 is an integration point between phototropin and phytochrome signalling

PKS4 plays a role in phytochrome signalling in red and far-red light (Schepens *et al*, 2008), as well as in phototropin signalling in blue light (Lariguet *et al*, 2006). How phytochromes modulate phototropism is not yet fully understood. It has been proposed that phyA exerts its role through regulation of nuclear gene expression such as *PKS1* and *RPT2* that are both positive regulators of phototropin signalling (Lariguet *et al*, 2006; Tsuchida-Mayama *et al*, 2010; Kami *et al*, 2012). Here, we report that PKS4L accumulation decreases with phytochrome activity (Figure 4). The phosphorylation status of PKS4 is thus controlled by the combined activities of phot1 and phytochromes, suggesting that PKS4 constitutes a molecular link between phot1 and phytochrome signalling in blue light. However, the mechanism by which the phytochromes control PKS4 phosphorylation remains to be determined. Phytochromes may control PKS4 phosphorylation by modulating the activity of a phosphatase and/or kinase in the cytosol. Alternatively, phytochromes may control the expression of genes coding for enzymes that modulate PKS4 phosphorylation.

PKS4L formation negatively regulates phototropism

It was previously shown that strong blue light treatments can negatively regulate phototropism, however, the underlying molecular mechanisms are unknown (Janoudi and Poff, 1991). Our data suggest that light-dependent PKS4 phosphorylation may contribute to this mechanism with PKS4L playing a negative role in phototropism. The increase in PKS4L accumulation observed in phytochrome-deficient mutants occurs at low blue light intensities where phytochromes are known to promote phototropic curvature (Parks *et al*, 1996; Janoudi *et al*, 1997; Lariguet and Fankhauser, 2004). In addition, the decrease in PKS4L accumulation following a red light pre-treatment also coincides with the phytochrome-mediated enhancement of phototropism (Liscum and Briggs, 1996; Parks *et al*, 1996; Figure 4B). More importantly support for a model where PKS4L functions to negatively regulate phototropism comes from our physiological experiments. Enhancement of the accumulation of PKS4L by increasing the intensity of blue light perceived by the plants or by pharmacological treatment involving the protein phosphatase inhibitor CN (Figure 8) leads to reduced phototropic curvature in WT but not in the *pks4* mutant. Cantharidin treatment enhances phot2-mediated phototropism, but inhibits phot1-mediated phototropism (Pedmale and Liscum, 2007; Tseng and Briggs, 2010) (this study), implying that phosphatase activity has an antagonistic role in phot1 and phot2 signalling. PKS4 phosphorylation status is controlled by a PP2A (Figure 4) while phot1-dependent NPH3 dephosphorylation involves PP1 activity (Pedmale and Liscum, 2007). Phot1 signalling is thus regulated by multiple phosphatases to coordinate phototropic signalling. NPH3 dephosphorylation is proposed to be essential for phototropism (Pedmale and Liscum, 2007). On the other hand, PKS4 phosphorylation may constitute part of a negative feedback loop to prevent excessive phototropism or serve as a delay mechanism. This situation would then be similar to NPH3 dephosphorylation except that PKS4 is first phosphorylated by phot1 in response to light. Understanding how these early phosphorylation events impact those associated with controlling polar auxin transport are important questions for the future.

The role of phot-mediated phosphorylation during plant growth

Phototropins control a variety of physiological and growth responses throughout the life cycle of plants that can all be linked to the maximization of their photosynthetic potential (Christie, 2007). Such responses are particularly important during the early phases of plant development when carbon is a major factor limiting plant growth (Pantin *et al*, 2011). Among the phot-mediated responses members of the PKS family are specifically required for root and hypocotyl phototropism, leaf flattening and positioning (de Carbonnel *et al*, 2010). Phototropin-mediated phosphorylation is essential for all tested phot responses in Arabidopsis (Inoue *et al*, 2008a, 2011). Here, we studied the role of PKS4 phosphorylation during young seedling development, as our previous investigation showed that PKS4 has a selective role in the control of hypocotyl tropic responses (Lariguet *et al*, 2006; Schepens *et al*, 2008; de Carbonnel *et al*, 2010). Similarly a role for ABCB19, another recently identified substrate of phot1, is currently not known for phot-mediated responses beyond

phototropism (Christie *et al*, 2011). It will be interesting to determine how phot-mediated phosphorylation events control plant growth later in development.

Materials and methods

Plant material and growth conditions

The Columbia (Col-O) ecotype of *A. thaliana* was used as the WT. All of the following mutant alleles were in the Col-O background: *pks4-1* (Lariguet *et al*, 2006), *phot1-5* (Huala *et al*, 1997), *phot2-1* (Kagawa *et al*, 2001), *cry1cry2* (Duek and Fankhauser, 2003) and *nph4* (Liscum and Briggs, 1996). The mutant *phyabcde* was in *ft* background (Strasser *et al*, 2010). Transgenic lines overexpressing PINOID, or expressing wild-type or mutated versions of phot1 were described elsewhere (Geldner *et al*, 2003; Cho *et al*, 2007; Inoue *et al*, 2008a). Seeds were surface-sterilized, plated and treated as described (Lariguet and Fankhauser, 2004). For protein extraction, 50 seeds were sown for each time point. For CN and hypocotyl curvature measurement, seeds were sown on nylon mesh (160 μm, Micropore) disposed on the surface of the plate. Seedlings were grown for 3 days in darkness at 22°C before the indicated treatment. Light intensities were determined with an International Light IL1400A photometer (Newburyport, MA) equipped with an SEL033 probe with appropriate light filters.

CN and λ-phosphatase treatments

Nylon meshes with 3-day-old etiolated seedlings were transferred onto freshly prepared plates supplemented by 0, 0.1, 1 or 10 μM cantharidin (Sigma) and 1% DMSO, 1 h before indicated light treatment. λ-Phosphatase treatment was performed as described in Lorrain *et al* (2008).

SDS–PAGE and immunoblot analysis

Total proteins (100 μl 2X Laemmli buffer for 20 mg fresh weight; 10 μg per lane) were separated on 8% SDS/PAGE gels and transferred onto nitrocellulose with 100 mM CAPS pH 11 + 10% (v/v) ethanol. The blots were probed with anti-DET3, anti-phot1 (Lariguet *et al*, 2006). Polyclonal anti-PKS4 antibodies were raised as follow: *PKS4 full* cDNA sequence was cloned into pHIS8-3 in frame with N-terminal 8-histidine (His) tag (to generate pIS25). His-PKS4 recombinant proteins were produced in *E. coli* by inducing gene expression with 0.2 mM IPTG for 4 h at 37°C. PKS4 proteins were purified from inclusion bodies and used to immunize rabbits (Eurogentec). After three boosts, the serum of one rabbit was retrieved and polyclonal antibodies specific to PKS4 were obtained by affinity purification (using purified GST-PKS4 proteins). Anti-PKS4 antibodies were used at a 1/500 dilution in PBS, 0.1% Tween-20, and 5% non-fat milk. Chemiluminescence signals were generated using Immobilon Western HRP Substrate (Millipore). Signals were captured with a Fujifilm ImageQuant LAS 4000 mini CCD camera system and quantifications were performed with ImageQuant TL software (GE Healthcare).

In-vitro phosphorylation assay

P1L2K polypeptides of the wild-type and the D788A mutant were prepared after the procedures reported previously (Okajima *et al*, 2011). The phot1 polypeptides were incubated with each PKS4 polypeptide or GST tag at 20°C for 30 min in a kinase reaction buffer containing 20 mM Tris–HCl (pH 7.8), 100 mM NaCl, 1 mM Na_2EGTA, 10% (w/v) glycerol, 20 μM ATP and 74 kBq of (γ-^{32}P) ATP in the presence of 10 mM $MnCl_2$. The effect of blue light on phosphorylation was measured by irradiation with a blue LED illuminator using mock irradiation as a control. Reaction was stopped by adding an SDS–PAGE sample buffer followed by boiling for 3 min. The samples were run on SDS–PAGE and then the proteins were blotted onto PVDF membrane. After Coomassie staining, phosphorylation of the proteins was visualized with imaging plate (Fujifilm) and a STORM scanner (GE Healthcare).

Hypocotyl growth measurement

Two-day-old etiolated seedlings (WT, *pks4*, *phot1*) were irradiated with 0.5, 10 or 100 $\mu mol\,m^{-2}\,s^{-1}$ blue light from above. Pictures were taken every 15 min, during 90 min before and after light treatment, using an Infra Red CCD Camera system. Hypocotyl growth rates were deduced from hypocotyl tips coordinates that were determined using HypoPhen software (http://www2.unil.ch/cbg/index.php?title=HypoPhen; Kami *et al*, 2012). Mean, standard error and Student's *t*-test were performed on 20 seedlings.

Hypocotyl curvature

Seedlings were grown on vertically orientated plates for 3 days in darkness at 22°C. For continuous light treatment, seedlings were irradiated with 0.1 $\mu mol\,m^{-2}\,s^{-1}$ unilateral blue light for 10 h. For pulse-light irradiation, seedlings were first sensitized by irradiation with 1800 μmol red light and kept in darkness for 90 min, and irradiated with a 10-min pulse of blue light at the indicated fluence rate and then kept in darkness at 22°C for 4 h. Seedlings with cotyledons in the opposite side of the incident light source were selected for measurement. Angles formed by the hypocotyl relative to vertical were measured with the NIH image software. Means, standard errors and Student's *t*-test were performed on 20 seedlings minimum.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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