Conditional Involvement of CONSTITUTIVE PHOTOMORPHOGENIC1 in the Degradation of Phytochrome A$^{1[W][OA]}$

Dimitry Debrieux$^2$, Martine Trevisan, and Christian Fankhauser*

Center for Integrative Genomics, Faculty of Biology and Medicine, University of Lausanne, CH–1015 Lausanne, Switzerland

All higher plants possess multiple phytochrome photoreceptors, with phytochrome A (phyA) being light labile and other members of the family being relatively light stable (phyB–phyE in Arabidopsis [Arabidopsis thaliana]). phyA also differs from other members of the family because it enables plants to deetiolate in far-red light-rich environments typical of dense vegetational cover. Later in development, phyA counteracts the shade avoidance syndrome. Light-induced degradation of phyA favors the establishment of a robust shade avoidance syndrome and was proposed to be important for phyA-mediated deetiolation in far-red light. phyA is ubiquitylated and targeted for proteasome-mediated degradation in response to light. Cul1 and the ubiquitin E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) have been implicated in this process. Here, we systematically analyze the requirement of cullins in this process and show that only CULLIN1 plays an important role in light-induced phyA degradation. In addition, the role of COP1 in this process is conditional and depends on the presence of metabolizable sugar in the growth medium. COP1 acts with SUPPRESSOR OF PHYTOCHROME A (SPA) proteins. Unexpectedly, the light-induced decline of phyA levels is reduced in spa mutants irrespective of the growth medium, suggesting a COPI-independent role for SPA proteins.

All living organisms need to perceive and respond to changes in the environment in order to adapt their growth and development to fluctuating conditions. This is particularly important for sessile organisms such as plants, which cannot seek for a better place in response to adverse environmental conditions. Plants are very sensitive to changes in the light environment, which they monitor with a battery of photoreceptors enabling them to sense wavelengths from UV-B to near infrared (Chen et al., 2004; Jenkins, 2009; Kami et al., 2010; Rizzini et al., 2011). The red and far-red light-absorbing phytochromes play key roles throughout the life cycle of plants, controlling a multitude of physiological responses including seed germination, seedling deetiolation, shade avoidance, and the transition to reproductive growth (Franklin and Quail, 2010; Kami et al., 2010).

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2 Present address: Alere GmbH, Moosacherstrasse 14, CH–8804 Au-Wädenswil, Switzerland.

* Corresponding author; e-mail christian.fankhauser@unil.ch.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Christian Fankhauser (christian.fankhauser@unil.ch).

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primarily controlled by light-stable phytochromes and inhibited by phyA (Robson et al., 1996; Franklin and Quail, 2010). Despite the importance of light-induced phyA degradation, the molecular mechanism underlying this crucial regulatory event is still poorly understood.

The decreased phyA levels in the light are due to reduced PHYA transcription and to proteasome-mediated degradation of the light-activated photoreceptor (Shanklin et al., 1987; Jabben et al., 1989; Cantón and Quail, 1999; Sharrock and Clack, 2002; Seo et al., 2004). The reduced stability of phyA in the light is partly due to the change in subcellular localization of the light-activated photoreceptors. In the dark, phyA is cytosolic, while upon light perception it is imported into the nucleus, where the photoreceptor is less stable (Debrieux and Fankhauser, 2010). However, phyA is also degraded in response to light when the protein remains in the cytosol, suggesting a multilevel control of phyA stability (Debrieux and Fankhauser, 2010). Proteasome-mediated protein degradation is a prominent mechanism controlling the abundance of numerous proteins in Arabidopsis, with 5% of its genome predicted to encode proteins involved in this process (Hellmann and Estelle, 2002; Hotton and Callis, 2008). Proteasome substrates are first marked with polyubiquitin chains, which are covalently attached to Lys residues by ubiquitin ligases. E3 ligases play a crucial role in this process because they control the substrate specificity of the ubiquitylation reaction (Hotton and Callis, 2008). Several classes of these enzymes exist in plants and animals, including multimeric E3 ligases (Hotton and Callis, 2008; Biedermann and Hellmann, 2011). A major type of multimeric E3 is the Cullin-RING Ligase type that is characterized by a Cullin, a RING finger protein called RING BOX1 (RBX1), and at least one additional component important to define substrate specificity (Hotton and Callis, 2008; Biedermann and Hellmann, 2011). In Arabidopsis, two genes code for RBX1 proteins, but only a single one is strongly expressed in all tissues (Gray et al., 2002; Lechner et al., 2002). Among the CULLIN genes present in the Arabidopsis genome, CUL1, CUL3a, CUL3b, and CUL4 have been characterized (Figueroa et al., 2005; Thomann et al., 2005; Bernhardt et al., 2006; Chen et al., 2006). CUL1 is part of SKP1 CULLIN F-Box (SCF)-type E3 ligase complexes, which also comprise an F-box protein and SKP1. CUL3 makes a complex with BTB/POZ domain proteins, while CUL4 acts with DDB1 and WD40 domain proteins such as CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) and SUPPRESSOR OF PHYTOCHROME A (SPA; Schwechheimer and Calderon Villalobos, 2004; Figueroa et al., 2005; Thomann et al., 2005; Bernhardt et al., 2006; Hotton and Callis, 2008; Chen et al., 2010a; Biedermann and Hellmann, 2011; Lau and Deng, 2012).

The ubiquitin E3 ligase COP1 is involved in the light-induced degradation of phyA (Seo et al., 2004; Saijo et al., 2008). Interestingly, phyA levels are also elevated in light-grown cul1 seedlings (Quint et al., 2005; Moon et al., 2007; Gilkerson et al., 2009). Given that COP1 acts in a CUL4 complex rather than in a CUL1-based SCF-type ubiquitin ligase complex, this suggests that multiple E3 ligases contribute to the control of phyA stability (Chen et al., 2010a; Lau and Deng, 2012). Finally, the HEMERA protein that interacts with both phyA and phyB has also been implicated in the degradation of phyA in the nucleus (Chen et al., 2010b; Galvão et al., 2012). As HEMERA shares some homology with the RAD23 protein, it could be involved in linking multiquabitinated phyA to the proteasome (Chen et al., 2010b). We thus decided to analyze the requirement of cullin-based E3 ligases systematically and found that only CUL1 is needed for light-induced phyA degradation, while the role of COP1 is conditional and depends on the presence of metabolizable sugar in the medium. Interestingly, SPA proteins, which are proposed to act with COP1 (Lau and Deng, 2012), are required for phyA degradation in the light irrespective of the presence of Suc. These results indicate a primary importance for CUL1 in phyA degradation and suggest that SPA proteins may act independently of COP1.

RESULTS

The Regulation of phyA Abundance in Seedlings Transferred into the Light Depends on Cullin-Based E3 Ligases

phyA has been shown to be ubiquitylated in the light, and its degradation is inhibited by proteasome inhibitors (Jabben et al., 1989; Seo et al., 2004; Debrieux and Fankhauser, 2010). In order to confirm that phyA degradation depends on ubiquitylation, we analyzed phyA protein levels in transgenic plants expressing a mutant form of ubiquitin that prevents the formation of the most common form of polyubiquitin chains (Lys-48): ubR48 (Schlögelhofer et al., 2006). The expression of this protein is controlled by dexamethasone (Dex; Schlögelhofer et al., 2006). Etiolated seedlings were treated with 100 μM Dex for 3 d in the dark prior to transfer into red light, and phyA protein accumulation was monitored during 6 h upon transfer into the light. This experiment showed that in the presence of ubR48, the light-induced decline in phyA levels was impaired (Fig. 1, A and B). In order to test whether this depends on cullin-based E3 ligases, we analyzed phyA protein levels in a transgenic line expressing an inducible RBX1 RNA interference (RNAi) construct, given that RBX1 is a common subunit of all known cullin-based E3 ligases (Lechner et al., 2002). When etiolated RBX1-RNAi seedlings grown for 3 d in the presence of Dex were transferred into red light, phyA levels remained higher than in the wild type, confirming an involvement of Dex in the control of phyA abundance in the light (Fig. 1, C and D).
In order to determine which cullin(s) in particular may be involved in the regulation of phyA stability, we analyzed phyA abundance in

\textit{cul1}, \textit{cul3a}, \textit{cul3b}, and \textit{cul4} mutants. Upon transfer into red light, phyA levels remained much higher in the \textit{cul1} mutant \textit{axr6-3} grown at 24°C, a restrictive temperature for this temperature-sensitive allele of \textit{cul1}. Our quantitative western-blot analysis thus confirmed a previous report and showed that the apparent half-life of phyA in \textit{axr6-3} is more than 3.5 h compared with about 90 min in the wild type (Fig. 2; Quint et al., 2005). In a previous study, the role of CUL3 was analyzed in the Wassilewskija (Ws) ecotype, and it was concluded that phyA degradation is normal in \textit{cul3a-1} (Dieterle et al., 2005). We confirmed this finding in Ws (Supplemental Fig. S1) and tested the role of CUL3 in Columbia (Col-0), as all other mutants tested here are in that ecotype. The light-induced decline in phyA levels was also normal in \textit{cul4} mutants (Fig. 3). It is important to point out that this might be due to the relatively weak nature of the \textit{cul4} allele analyzed here (Bernhardt et al., 2006).

\textbf{COP1 Plays a Conditional Role in the Regulation of phyA Abundance}

The normal light-induced decline of phyA in \textit{cul4} mutants was surprising, given that phyA degradation was reported to depend on COP1 and that COP1 in complex with SPA proteins is part of a CUL4-based E3 ligase (Seo et al., 2004; Chen et al., 2010a; Lau and Deng, 2012). We thus analyzed phyA protein levels in etiolated seedlings transferred into red light in \textit{cop1-4} and in a mutant lacking three of the four SPA genes present in Arabidopsis (\textit{spa1spa2spa3} triple mutant; Laubinger et al., 2004; Saijo et al., 2008; Lau and Deng, 2012). The light-induced decline in phyA levels was strongly attenuated in \textit{spa1spa2spa3}, while, surprisingly, in the \textit{cop1-4} mutant we detected no effect on

\textbf{Figure 1.} Cullin-based ubiquitin E3 ligases are required for the reduction of phyA levels in the light. Total protein extracts from 3-d-old etiolated Col-0, \textit{RBX1} RNAi (\textit{rbx1}), and ubR48-expressing seedlings transferred into red light (R; 50 \(\mu\text{mol m}^{-2} \text{s}^{-1}\)) for the indicated amounts of time were separated on 8% SDS-PAGE gels, western blotted, and probed with anti-phyA and anti-DET3 antibodies. Seedlings were grown on one-half-strength MS without Suc. A and C, Representative western blots. B and D, Quantification of phyA levels. Results are expressed relative to the dark level of each genotype; data are means of biological triplicates ± so.
phyA protein levels (Fig. 4, A–D). Similarly the light-regulated abundance of phyA was normal in cop1-6, another viable COP1 allele (data not shown).

All our experiments were performed with seedlings grown on one-half-strength Murashige and Skoog medium (MS) in the absence of Suc because a number of studies have shown that Suc interferes with phyA signaling (Dijkwel et al., 1997). Therefore, we decided to check whether the involvement of COP1 in the control of phyA abundance might be conditional by repeating this experiment in the presence of 2% Suc, which is used in the growth medium in some laboratories. Interestingly, under these conditions, the decline in phyA levels was impaired in cop1-4 (Fig. 4, E and F), confirming previous results that we presume were obtained in the presence of Suc in the growth medium (Seo et al., 2004). In order to determine whether this is a metabolic or an osmotic effect of Suc, we repeated the experiment in the presence of 3-O-methyl-D-Glc, a nonmetabolizable Glc analog, and found that on this medium the regulation of phyA abundance was unaffected in cop1-4 (Fig. 4G). These data confirmed that the role of COP1 in the regulation of phyA abundance is only detectable in the presence of a metabolizable source of Glc. Consistent with this hypothesis, phyA levels remained higher in etiolated seedlings transferred into red light in cop1-4 grown on maltose (data not shown). The conditional phenotype of cop1-4 in the regulation of phyA abundance prompted us to analyze phyA protein levels in cul1 and cul4 in the presence of 2% Suc (Fig. 5). Somewhat surprisingly (see “Discussion”), the light-induced reduction in phyA levels remained wild type in cul4 grown on 2% Suc. The role of CUL1 in the regulation of phyA levels was somewhat attenuated in the presence of Suc (Fig. 5).

In order to verify that the effects of COP1 and CUL1 were not due to the light-regulated transcriptional decline of PHYA, we conducted a quantitative reverse transcription-PCR analysis comparing those mutants with the wild type. Three-day-old etiolated seedlings were grown on one-half-strength MS with or without Suc and either kept in the dark or transferred into red light for an additional 4 h before RNA extraction. In the presence or absence of Suc, light triggered a decline in PHYA levels (Fig. 6). Moreover, none of the tested genotypes significantly altered the transcriptional regulation of PHYA. Taken together, our data indicate a primary role for cullin1 in the light-induced degradation of phyA. Additionally, SPA proteins play a role in this process. Interestingly, the role of COP1 is conditional and depends on the presence of a metabolizable source of hexose in the growth medium. To further define the conditions where COP1 is involved in the degradation of phyA, we grew seedlings on soil and analyzed the light-induced phyA decline in wild-type, cop1-4, and cop1-6 seedlings. As previously observed on one-half-strength MS without Suc (Fig. 4), phyA levels declined normally in soil-grown cop1 alleles transferred into red light (Fig. 7). We thus conclude that the requirement of COP1 for the degradation of phyA is restricted to specific conditions.

Figure 2. Cullin1 is important for the light-induced reduction of phyA abundance. Three-day-old etiolated seedlings of Col-0 or cul1/axr6-3 transferred into red light (R; 50 μmol m⁻² s⁻¹) for the indicated amounts of time were separated on 8% SDS-PAGE gels, western blotted, and probed with anti-phyA and anti-DET3 antibodies. Seedlings were grown on one-half-strength MS without Suc. A, Representative western blot. B, Quantification of phyA levels. Results are expressed relative to the dark level of each genotype; data are means of biological triplicates ± SD.

Figure 3. Normal light-induced decrease in phyA abundance in cul4-1. Total protein extracts from 3-d-old etiolated Col-0 and cul4-1 seedlings transferred into red light (R; 50 μmol m⁻² s⁻¹) for the indicated amounts of time were separated on 8% SDS-PAGE gels, western blotted, and probed with anti-phyA and anti-DET3 antibodies. Seedlings were grown on one-half-strength MS without Suc. A, Representative western blot. B, Quantification of phyA levels. Results are expressed relative to the dark level of each genotype; data are means of biological triplicates ± SD.
DISCUSSION

Although the importance of the light-induced decline in phyA levels has long been recognized, we still know relatively little about the molecular events underlying this regulation (Robson et al., 1996; Franklin and Quail, 2010; Rausenberger et al., 2011). Moreover, based on recent modeling and experimental data, light-induced degradation of phyA is an essential feature allowing this class of phytochromes to promote deetiolation in FR-rich environments (Rausenberger et al., 2011). In monocotyledons, light-regulated transcription of PHYA plays a more prominent role than in dicotyledons (Cantón and Quail, 1999). In addition, light-induced ubiquitination and degradation of phyA is a prominent mechanism to reduce phyA levels in...
numerous plant species (Jabben et al., 1989). The E3 ubiquitin ligase COP1 and the SPA proteins that associate with COP1 are implicated in light-induced phyA turnover (Seo et al., 2004; Saijo et al., 2008). These proteins form a multisubunit complex with CUL4-DDB1-RBX1 involved in the degradation of important regulators of light signaling (Chen et al., 2010a; Lau and Deng, 2012). Moreover, light-grown cul1 mutants also show higher phyA protein levels than the wild type (Quint et al., 2005; Moon et al., 2007; Gilkerson et al., 2009), suggesting that multiple cullin-based E3 ligases are involved in the control of phyA turnover.

To test this hypothesis, we determined phyA protein levels in etiolated seedlings transferred into red light by quantitative western-blot analysis in different cullin mutants. As reported previously, we confirmed that although phyA levels are normal in etiolated axr6-3 mutants, the decline in phyA abundance upon transfer into red light was considerably diminished in this cul1 allele (Quint et al., 2005; Fig. 2; Supplemental Fig. S2). Given that the light-regulated transcription of PHYA is normal in this mutant background, it is likely that this is due to reduced cullin1-based ubiquitylation of phyA in axr6-3 (Fig. 6). The analysis of phyA protein levels in several cul1 alleles suggests that CUL1 is primarily involved in the light to regulate phyA turnover, although based on qualitative analysis, it was proposed that phyA levels are also altered in etiolated cul1-6 (Quint et al., 2005; Moon et al., 2007; Gilkerson et al., 2009). Although we present all our data relative to the dark level of each genotype (Figs. 1–5), we also compared phyA levels in etiolated mutants with Col-0 on those blots and found no significant difference (Supplemental Fig. S2).

Normal light-induced degradation of phyA was previously reported in a cul3a allele in the Ws ecotype (Dieterle et al., 2005). As Arabidopsis contains two CUL3 genes, we analyzed this in more detail (Dieterle et al., 2005; Figueroa et al., 2005; Thomann et al., 2005). The light-induced decline in phyA levels was normal in both cul3a alleles tested (one in Ws and the other in Col-0, as all other mutants tested here) and in a T-DNA insertion allele in cul3b (Supplemental Fig. S1). These cul3 single mutants develop relatively normally, although cul3a mutants have some phenotypes related to light sensing (Dieterle et al., 2005). In contrast, the cul3a cul3b double mutant is embryonic lethal, preventing us from analyzing the stability of phyA in the absence of CUL3 (Dieterle et al., 2005). However, we analyzed phyA levels in a double mutant combining a hypomorphic cul3a allele with a cul3b null (Thomann et al., 2009), a background in which we also observed a normal light regulation of phyA abundance (Supplemental Fig. S1). We thus conclude

Figure 5. The effect of Suc in the growth medium on light-regulated phyA abundance in cul1 and cul4. Total protein extracts from 3-d-old etiolated Col-0, cul1, and cul4 seedlings grown on one-half-strength MS with 2% Suc transferred into red light (R; 50 μmol m⁻² s⁻¹) for the indicated amounts of time were separated on 8% SDS-PAGE gels, western blotted, and probed with anti-phyA and anti-DET3 antibodies. A and C, Representative western blots. B and D, Quantification of phyA levels. Results are expressed relative to the dark levels of each genotype; data are means of biological triplicates ± so.
that CUL3 does not play a major role in phyA degradation under our experimental conditions.

The role of COP1 in the light-induced degradation of phyA prompted us to analyze phyA levels in a cul4 mutant. We used the cul4-1 allele that has reduced CUL4 protein levels but is not a null allele (Bernhardt et al., 2006). Unfortunately, we could not test the involvement of CUL4 with stronger alleles because such mutants are lethal (Chen et al., 2006). Upon transfer of etiolated seedlings into red light, phyA protein levels in cul4-1 were not significantly different from the wild type (Fig. 3). This result is somewhat surprising, given that COP1 has been shown to act as part of a CUL4 SCF-type E3 ligase (Seo et al., 2004; Chen et al., 2010a). We thus analyzed phyA levels in cop1-4 to determine whether under our experimental conditions COP1 plays an important role in phyA degradation. Interestingly, when seedlings were grown on one-half-strength MS without Suc, the light-induced decline in phyA abundance was unaffected in cop1-4 and in cop1-6 (Fig. 4; data not shown).

The absence of a phenotype in cul4-1 and viable cop1 alleles may be due to residual activity present in these alleles that are not null (Seo et al., 2004; Bernhardt et al., 2006). Strong cop1 alleles are seedling lethal, so we used the fusca-colored seeds from a heterozygous cop1-5 plant for this experiment (Ang and Deng, 1994). Unfortunately these seedlings developed so poorly on one-half-strength MS without Suc that we could not perform the experiment. However, since cop1-4 was previously shown to be defective in phyA turnover, we decided to investigate whether this phenotype might be conditional. By analyzing phyA degradation in seedlings grown on different media, we found that cop1-4 only showed a phenotype when grown in the presence of a metabolizable source of hexose (Fig. 4; data not shown). However, we observed a normal light-induced decline in phyA abundance in soil-grown cop1-4 and cop1-6, suggesting that the role of COP1 in the regulation of phyA turnover is restricted to specific conditions (Fig. 7). We were unable to detect an effect of sugar on phyA degradation in a wild-type background or in cul4-1 (Figs. 3 and 5; data not shown). As indicated above, this could be due to the residual CUL4 activity in cul4-1 (Seo et al., 2004; Bernhardt et al., 2006). However, it should be pointed out that weak cul4 alleles have a deetiolated phenotype in darkness that is consistent with the role of CUL4 in the degradation of other COP1 substrates such as HY5 (Bernhardt et al., 2006; Chen et al., 2006). Alternatively, one could propose that at least in some conditions, COP1 may act as a stand-alone E3 ligase, an activity that it displays in vitro (Seo et al., 2004; Bernhardt et al., 2006).

Given that SPA proteins also belong to a protein complex with COP1 and CUL4 and were shown to be involved in phyA degradation (Saijo et al., 2008; Chen et al., 2010a), we analyzed phyA protein levels in spa1spa2spa3 triple mutants and found that in this mutant background, phyA degradation was considerably impaired (Fig. 4). Based on the current literature, these results are surprising for two reasons. First, an analysis of cryptochrome2 (cry2) degradation has shown that for this light-labile photoreceptor, COP1 and SPA proteins are both necessary to regulate its turnover (Weidler et al., 2012). However, it should be pointed out that the exact role of COP1 in the regulation of cry2 abundance is not fully solved, as cry2 degradation is still observed in cop1 null alleles (Shalitin et al., 2002). Second, our results suggest that SPA proteins may act independently of COP1 and that COP1 is not essential to regulate phyA protein abundance under all experimental conditions. Importantly, the strong phyA degradation phenotype of spa1spa2spa3 is consistent with previous studies that have shown that the light-grown phenotype of spa mutants depends on phyA (Hoecker et al., 1998; Laubinger and Hoecker, 2003; Laubinger et al., 2004). Interestingly, in

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Effects of COP1 and CUL1 on PHYA transcript levels. Expression levels of PHYA in 3-d-old etiolated Col-0, cop1-4, and cul1 seedlings grown on one-half-strength MS with or without 2% Suc either kept in the dark or exposed to 4 h of red light (50 μmol m$^{-2}$ s$^{-1}$) were analyzed by reverse transcription followed by real-time PCR. EF1 and YLS8 were used as housekeeping genes. Data are normalized to PHYA in etiolated wild-type plants and correspond to means ± so of three independent biological replicates with technical triplicates for each sample.

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Normal phyA degradation in soil-grown cop1 mutants. Total protein extracts from 3-d-old etiolated Col-0, cop1-4, and cop1-6 seedlings grown on soil transferred into red light (R; 50 μmol m$^{-2}$ s$^{-1}$) for the indicated amounts of time were separated on 8% SDS-PAGE gels, western blotted, and probed with anti-phyA and anti-DET3 antibodies.
Control of phyA Degradation

**Materials and Methods**

**Growth Conditions**

Arabidopsis (Arabidopsis thaliana) seeds were surface sterilized by soaking for 5 min in 70% ethanol plus 0.05% Triton X-100, followed by an incubation of 10 to 15 min in 100% ethanol. Seeds were plated on one-half-strength MS (Duchefa Biochemie) plus 0.8% (w/v) Phytagar (Gibco BRL) in petri dishes for 5 min in 70% ethanol plus 0.05% Triton X-100, followed by an incubation of 10 to 15 min in 100% ethanol. Seeds were plated on one-half-strength MS medium; this information is specified in the figure legends. The plates were stored at 4°C in the dark during 3 d for stratification, followed by a 6-h white light (100 μmol m⁻² s⁻¹) treatment to induce germination. After this step, the plates were wrapped in aluminum foil and placed in a Phytocon 20°C for 3 d to produce etiolated seedlings and/or put in the desired light treatment (out light from light-emitting diode sources with a maximum emission peak at 670 nm). The following genotypes were used in this study. Col-0 was used as a wild type, and all mutants are in this ecotype: *cop1-1, cop1-6* (Seo et al., 2004), *ubr46*, a line expressing a ubiquitin R46K mutant in a Dex-inducible manner (Schloegelhofer et al., 2006), a Dex-inducible RNAi *rbx1* mutant (Lechner et al., 2002), *axr6-3*, a temperature sensitive *cul1* allele (Quint et al., 2005), *cul3a-2* and *cul3b-1* (Thomann et al., 2005), *cul3a-3* and *cul3b-1* (Thomann et al., 2009), *cul4-1* (Bernhardt et al., 2006), and the *spa1-7;spa2-1;spa3-1* triple mutant (Laubinger and Hoecker, 2003; Fittinghoff et al., 2006). *cul3a-1* is in the Ws ecotype and was compared with its isogenic parent (Thomann et al., 2005). For Dex induction, we used 10 μg/ml of Dex directly supplemented to the one-half-strength MS.

**Quantitative Western-Blot Analysis**

Quantitative western-blots were performed essentially as described (Trupkin et al., 2007). For each time point, we used 50 etiolated seedlings, which were exposed for various amounts of time to continuous red light (50 μmol m⁻² s⁻¹). Total protein extraction was performed by grinding the seedlings with blue pestles in Eppendorf tubes in the presence of boiling 2X SDS-PAGE sample buffer. Proteins were separated on 8% acrylamide SDS-PAGE gels and western blotted onto nitrocellulose (Bio-Rad). The membranes were blocked overnight with the Odyssey blocking buffer (Li-Cor Biosciences; catalog no. 927-40010). The membranes were probed with a mouse monoclonal antibody directed against phyA (A4003; Shinomura et al., 1996) or a rabbit polyclonal antibody against DET3 (Schumacher et al., 1999) diluted 1:5,000 or 1:10,000, respectively. After two washing steps of 10 min, the membrane was incubated for 30 min with the secondary antibodies Alexa Fluor 680 goat anti-mouse (Molecular Probes) or IRDye 800 conjugated goat anti-rabbit (Rockland), both diluted 1:5,000. The signals were visualized using the Odyssey infrared imaging system (Li-Cor Biosciences) according to the manufacturer’s instructions. The data were normalized by dividing the signal intensity of phyA by the signal intensity of DET3 in each lane.

**Analysis of Gene Expression**

Total RNA was extracted from 3-d-old dark-grown seedlings exposed to red light (50 μmol m⁻² s⁻¹) during 0 or 4 h using the Qiagen RNeasy Plant Mini Kit. These samples were treated with Qiagen DNase and reverse transcribed using the SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with the Power SYBR Green PCR master mix from Applied Biosystems using the ABI Prism 7900 HT Sequence Detection System according to the manufacturer's instructions. For the relative quantification of the gene expression, we used qBase software for the management and automated analysis of real-time PCR (http://medgen.ugent.be/qbase). Each reaction was performed in triplicate using a primer concentration of 300 nM. EF1α (A5G60390) and YLS8 (A5G68290) were used as housekeeping genes. The following primers were used: EF1α (reverse, 5'-ATGAAGACACCTCCTTGATGATTTC-3') and YLS8 (reverse, 5'-CTACAGACAGCTGGTCCATCCTG-3'). Each primer was chosen to be specific for the resistance cassette in the ethylene insensitive 3 transcription factor, suggesting that sugar metabolism may regulate the stability of multiple proteins (Yanagisawa et al., 2003). Further work is required to understand how metabolism affects phyA levels and thus potentially the light sensitivity of Arabidopsis.

**Supplemental Data**

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

**Supplemental Figure S1.** Cullin3 is dispensable for light-induced phyA degradation.

**Supplemental Figure S2.** phyA levels in etiolated seedlings.

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