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Conditional involvement of constitutive photomorphogenic1 in the degradation of phytochrome A..

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Abstract

All higher plants possess multiple phytochrome (phy) photoreceptors with phyA being light-labile and other members of the family being relatively light stable (phyB-phyE in Arabidopsis). phyA also differs from other members of the family because it enables plants to de-etiolate in far-red (FR) 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 de-etiolation in FR light. phyA is ubiquitylated and targeted for proteasome-mediated degradation in response to light. Cullin1 and the ubiquitin E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (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 media. COP1 acts with SUPPRESSOR OF PHYA (SPA) proteins. Unexpectedly the light-induced decline of phyA levels is reduced in *spa* mutants irrespective of the growth media suggesting a COP1-independent role for SPA proteins.

Keywords

Phytochrome, phyA, proteasome, cullin, COP1, sucrose

Introduction

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 wavelength from UV-B to near IR (Chen et al., 2004; Jenkins, 2009; Kami et al., 2010; Rizzini et al., 2011). The red, far-red absorbing phytochromes play key roles throughout the life cycle of plants controlling a multitude of physiological responses including seed germination, seedling de-etiolation, shade avoidance and the transition to reproductive growth (Franklin and Quail, 2010; Kami et al., 2010).

All higher plants possess multiple phytochromes that can be classified as light labile (phyA in Arabidopsis) and light stable (phyB-phyE in Arabidopsis) (Quail, 2002). Plant phytochromes are synthesized and assembled in the inactive red-light absorbing form known as Pr. Upon red light perception they are converted into the active, far-red –light absorbing conformer known as Pfr. Pfr will slowly revert to the inactive state in the absence of light (dark reversion) (Hennig et al., 1999), a reaction that is very fast in the presence of far-red (FR) light (Rockwell et al., 2006). Light-stable phytochromes predominantly control R/FR-reversible light responses while phyA has a specific mode of action allowing it to control seed germination and seedling de-etiolation even when only a very minor fraction of the photoreceptor is in its active Pfr conformation (Quail, 2002; Franklin and Quail, 2010; Kami et al., 2010). Such

conditions are encountered under deep vegetational cover and it has been argued that the appearance of phyA class phytochromes has provided a competitive advantage to flowering plants when plant cover on earth became important (Mathews, 2006). A mechanism explaining how phyA triggers de-etiolation in FR light has recently been proposed (Rausenberger et al., 2011). Interestingly light-induced degradation of phyA is a theoretical requirement for the proposed model and is a long-known feature that distinguishes phyA from other phytochromes (Franklin and Quail, 2010; Rausenberger et al., 2011). Reducing levels of phyA in light-grown seedlings is also important to allow a proper shade avoidance response that is 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 are 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; Canton 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 lysine residues by ubiquitin ligases. E3 ligases play a crucial role in this process because they control 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 CRL (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 *CULLINs* 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 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 protein such as COP1 and SPA proteins (Schwechheimer and Calderon Villalobos, 2004; Figueroa et al., 2005; Thomann et al., 2005; Bernhardt et al., 2006; Hotton and Callis, 2008; Chen et al., 2010; 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., 2010; 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., 2010; Galvao et al., 2012). As HEMERA shares some homology with the RAD23 protein it could be involved in linking multi-ubiquitinated phyA to the proteasome (Chen et al., 2010). 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 media. 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 sucrose. 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 poly-ubiquitin chains (Lys 48): ubR48 (Schlogelhofer et al., 2006). The expression of this protein is controlled by dexamethasone (Dex) (Schlogelhofer et al., 2006). Etiolated seedlings were treated with 100 μM Dex for three days in the dark prior to transfer into red light and phyA protein accumulation was monitored during 6 hours upon transfer into the light. This experiment showed that in the presence of ubR48 the light-induced decline in phyA levels was impaired (Figure 1A 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* 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 three days in presence of Dex were transferred into red light phyA levels remained higher than in the wild type confirming an involvement of cullin-based E3 in the control of phyA abundance in the light (Fig. 1C, D).

In order to determine which cullin(s) in particular may be involved in the regulation of phyA stability we analyzed phyA abundance in *cul1*, *cul3a*, *cul3b* and *cul4* mutants. Upon transfer into red light phyA levels remained much higher in the *cul1* mutant *axr6-3* grown at 24°C, a restrictive temperature for this temperature-sensitive allele of *cul1*. Our quantitative western blot analysis thus confirms a previous report and showed that the apparent half-life of phyA in *axr6-3* is more than 3 ½ hours compared to about 90 minutes in the wild type (Figure 2) (Quint et al., 2005). In a previous study the role of CUL3 was analyzed in the Ws ecotype and concluded that phyA degradation is normal in *cul3a-1* (Dieterle et al., 2005). We confirmed this finding in Ws (Supplemental Figure S1) and tested the role of CUL3 in Col-0 as all other mutants tested here are in this ecotype. The light-induced decline in phyA levels was also normal in *cul3a-2*, a null allele in Col-0 (Supplemental Figure S1). As a double mutant null for both CUL3a and CUL3b is embryo lethal (Dieterle et al., 2005; Figueroa et al., 2005; Thomann et al., 2005), we analyzed phyA levels in the viable *cul3a-3 cul3b-1* mutant that combine a hypomorphic *CUL3a* allele with a *CUL3b* null (Thomann et al., 2009). As in the other cullin3 mutant tested, the light-induced decline in phyA levels was normal in *cul3a-3 cul3b-1* (Supplemental Figure S1). Finally we analyzed phyA levels in a *cul4* mutant, which under our experimental conditions did not affect light-regulated phyA abundance (Figure 3A, B). It is important to point out that this might be due to the relatively weak nature of the *cul4* allele analyzed here (Bernhardt et al., 2006).

COP1 plays a conditional role in the regulation of phyA abundance

The normal light-induced decline of phyA in *cul4* mutants was surprising given that phyA degradation was reported to depend on COP1 and COP1 in complex with SPA proteins is part of a CUL4-based E3 ligase (Seo et al., 2004; Chen et al., 2010; Lau and Deng, 2012). We thus analyzed phyA protein levels in etiolated seedlings transferred into red light in *cop1-4* and in a mutant lacking 3 of the 4 *SPA* genes present in Arabidopsis (*spa123* 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 *spa123* while surprisingly in the *cop1-4* mutant we detected no effect on phyA protein levels (Figure 4A-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 ½ strength MS in the absence of sucrose because a number of studies have shown that sucrose interferes with phyA signaling (Dijkwel et al., 1997). We therefore 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% sucrose that is used in the growth media in some laboratories. Interestingly under these conditions the decline in phyA levels was impaired in *cop1-4* (Figure 4 E, F), confirming previous results that we presume were obtained in the presence of sucrose in the growth media (Seo et al., 2004). In order to determine whether this is a metabolic or an osmotic effect of sucrose we repeated the experiment in the presence of 3-O-CH_3-D-Glc a non-metabolizable Glc analog and found that on this media the regulation of phyA abundance was unaffected in *cop1-4* (Figure 4 G). These data confirmed that the role of COP1 on the regulation of phyA abundance is only detectable in the presence of a metabolizable source of glucose. 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% sucrose (Figure 5). Somewhat surprisingly (see discussion) the light-induced reduction in phyA levels remained wild type in *cul4* grown on 2% sucrose. The role of CUL1 in the regulation of phyA levels was somewhat attenuated in the presence of sucrose (Figure 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 RT-PCR analysis comparing those mutants to the wild type. 3-day-old etiolated seedlings were grown on ½ strength MS with or without sucrose and either kept in the dark or transferred into red light for an additional 4 hours before RNA extraction. In the presence or absence of sucrose light triggered a decline in *PHYA* levels (Figure 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. Moreover 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 media. 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 the wild type, *cop1-4* and *cop1-6* seedlings. As previously observed on ½ strength MS without sucrose (Figure 4), phyA levels declined normally in soil-grown *cop1* alleles transferred into red light (Figure 7). We thus conclude that the requirement of COP1 for the degradation of phyA is restricted to specific conditions.

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 de-etiolation in FR-rich environments (Rausenberger et al., 2011). In monocotyledons light-regulated transcription of *PHYA* plays a more prominent role than in dicotyledons (Canton 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., 2010; Lau and Deng, 2012). Moreover light-grown *cul1* mutants also shows 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.

In order 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) (Figures

2, 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* (Figure 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 (Figures 1-5) we also compared phyA levels in etiolated mutants to Col-0 on those blots and found no significant difference (Supplemental Figure 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 the other in Col-0, as all other mutants tested here) and in a T-DNA insertion allele in *CUL3b* (Supplemental Figure S1). These *cul3* single mutants develop relatively normally although *cul3a* mutants have some phenotypes related to light sensing (Dieterle et al., 2005). In contrast a *cul3a cul3b* double mutant is embryonic lethal preventing us from analyzing the stability of phyA in the absence of CULLIN3 (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 Figure 1). We thus conclude that CULLIN3 do not play a major role in phyA degradation under our experimental conditions.

The role of COP1 is 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 (Figure 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., 2010). 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 ½ strength MS without sucrose the light-induced decline in phyA abundance was unaffected in *cop1-4* and in *cop1-6* (Figure 4, data not shown).

The absence of phenotype in *cul4-1* and viable *cop1* alleles may be due to residual activity present in those alleles that are not null (Seo et al., 2004; Bernhardt et al., 2006). Strong *cop1* alleles are seedling lethal we thus attempted using the fusca-colored seeds from a heterozygous *cop1-5* for this experiment (Ang and Deng, 1994). Unfortunately these seedlings developed so poorly on ½ strength MS without sucrose that we could not make 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 (Figure 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 (Figure 7). We were unable to detect an effect of sugar on phyA degradation in a wild-type background or in *cul4-1* (Figures 3, 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 de-etiolated 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., 2010), we analyzed phyA protein levels in *spa1spa2spa3* triple mutants and found that in this mutant background phyA degradation was considerably impaired (Figure 4). Based on the current literature these results are surprising for two reasons. First an analysis of cryptochrome 2 (cry2) degradation has shown that for this light-labile photoreceptor COP1 and SPA proteins are both necessary to regulate its turn over (Weidler et al., 2012). It should however 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 *spa1spa2spa3* seedlings hypocotyl growth is inhibited by FR light indicating that phyA-mediated de-etiolation occurs when phyA degradation is impaired (Figure 4)(Balcerowicz et al., 2011). A detailed analysis of phyA degradation in *spa* mutants combined with the determination of the action spectra of phyA-mediated de-etiolation might allow an experimental validation of the proposed requirement for phyA degradation during the phyA FR-HIR (Rausenberger et al., 2011).

In contrast to *cop1* mutants, phyA degradation in *axr6-3* was less affected in the presence than in the absence of sucrose (Figures 2 and 5). This is interesting given that *axr6-3* is sugar hypersensitive (Quint et al., 2005). A link between sugar metabolism and the control of photoreceptor abundance is potentially of great interest, however the existence of such a link in a wild-type background remains to be identified. In rice phyA degradation is controlled by jasmonic acid (JA) levels (Riemann et al., 2009). How JA modulates the degradation of phyA is unknown but it is interesting to note that the *axr3-6*, which is impaired in phyA degradation is also hyposensitive to JA (Quint et al., 2005) (Figure 2). The link between phytochrome signaling and JA signaling has received quite some attention recently however in Arabidopsis it is unknown whether JA regulates phyA abundance (Moreno et al., 2009; Robson et al., 2010).

Taken together our data show that cullin1-based ubiquitin E3 ligases play a primary role in phyA degradation and in some conditions COP1 also contributes to the downregulation of phyA. In addition our work shows that degradation of phyA is modulated by the presence of metabolizable sugar (Figure 4). Interestingly a

regulation of protein turnover by sucrose was previously identified for the Ethylene Insensitive 3 (EIN3) 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 light sensitivity of Arabidopsis.

Materials and Methods

Growth conditions

Seeds were surface sterilized by soaking for 5 min in 70% ethanol + 0.05% Triton X-100, followed by an incubation of 10-15 min in 100% ethanol. Seeds were plated on ½ strength MS (Duchefa Biochemie) + 0.8% (w/v) Phytagar (Gibco BRL, Grand Island, N.Y, USA) in Petri dishes (42 x 35mm^2 x 20mm). In some experiments 2% sucrose was added to the media, this information is specified in the figure legends. The plates were stored at 4°C in the dark during 3 days for stratification followed by a 6 hour white light (100µmol $m^{-2}s^{-1}$) treatment to induce germination. After this step the plates were wrapped in aluminium foil and placed in phytotron (20°C) for three days to produce etiolated seedlings and/or put in the desired light treatment (Red light from LED sources with λmax 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, *cop1-4*, *cop1-6* (Seo et al., 2004), ubR48 a line expressing a ubiquitin R48K mutant in a dexamethasone (Dex) inducible manner (Schlogelhofer 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 cul3b-1* (Thomann et al., 2009), *cul4-1* (Bernhardt et al., 2006) and a *spa1-7spa2-1spa3-1* triple mutant (Laubinger and Hoecker, 2003; Fittinghoff et al., 2006). *cul3a-1* is in the Ws ecotype and was compared to its isogenic parent (Thomann et al., 2005). For Dex inductions we used 10µM that we directly supplemented to the ½ strength MS.

Quantitative western blot analysis (Li-Cor)

Quantitative western blots were performed essentially as described in (Trupkin et al., 2007). For each time point we used fifty etiolated seedlings, which were exposed for various amounts of time to continuous red (50 µmol m^{-2} s^{-1}) light. Total protein extract were performed by grinding the seedlings with blue pestles in Eppendorf tubes in 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 GmbH Cat n°927-40010). The membranes were probed with a mouse monoclonal antibody directed against phyA (AA001) (Shinomura et al., 1996) or a rabbit polyclonal antibody against DET3 (Schumacher et al., 1999) diluted 1/5000 and 1/10'000 respectively. After two washing steps of 10 minutes, the membrane was incubated for 30 minutes with the secondary antibodies Alexa Fluor 680 goat anti-mouse (Molecular probes) or IRDye 800 Conjugated Goat anti-rabbit (Rockland) both diluted 1/5000. The signals were visualized using the Odyssey instrument (Odyssey infrared imaging system, Li-Cor Biosciences, Lincon, Nebraska 68504 USA) according to the manufacturer's indications. 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-day-old dark-grown seedlings exposed to red light 50µmol m^{-2} s^{-1} during 0 or 4h, using a QIAGEN RNeasy Plant Mini Kit®. These samples were treated with QIAGEN DNAseI® 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

Systems according to the manufacturer's instructions. For the relative quantification of the genes we used the qBase software for management and automated analysis of real-time PCR (http://medgen.ugent.be/qbase). Each reaction was performed in triplicate using a primer concentration of 300nM. *EF1α* (At5G60390) and *YLS8* (At5G08290) were used as House keeping genes. The following primers were used:

EF1α (R-atg aag aca cct cct tga tga ttt c / F-tgg tgt caa gca gat gat ttg c)

YLS8 (R-ctc agc aac aga cgc aag ca / F-tca ttc gtt tcg gcc atg a)

PHYA (R-gca aac tag cgc gtt atg tc / F-ccg aac act ctt tcc gtt ac).

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Figure Legends

Figure 1: Cullin-based ubiquitin E3 ligases are required for the reduction of phyA levels in the light. Total protein extracts from 3-day-old etiolated Col-0, *RBX1* RNAi (*rbx1)* and ubR48 expressing seedlings transferred into red light (50 μmol m^{-2} s^{-1}) for the indicated amount of time were separated on 8% SDS-PAGE gels, western blotted and probed with anti phyA and anti DET3 antibodies. Seedlings were grown on ½ strength MS without sucrose. (A, C) Representative western blot. (B, D) Quantification of phyA levels. Results are expressed relative to the dark levels of each genotype; data are means of biological triplicates +/- SD.

Figure 2: Cullin1 is important for the light-induced reduction of phyA abundance. 3-day-old etiolated seedlings of Col-0 or *cul1/axr6-3* transferred into red light (50μmol m^{-2} s^{-1}) for the indicated amount of time were separated on 8% SDS-PAGE gels, western blotted and probed with anti phyA and anti DET3 antibodies. Seedlings were grown on ½ strength MS without sucrose. (A) Representative western blot. (B) Quantification of phyA levels. Results are expressed relative to the dark levels 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-day-old etiolated Col-0 and *cul4-1* seedlings transferred into red light (50μmol m^{-2} s^{-1}) for the indicated amount of time were separated on 8% SDS-PAGE gels, western blotted and probed with anti phyA and anti DET3 antibodies. Seedlings

were grown on ½ strength MS without sucrose. (A) Representative western blot. (B) Quantification of phyA levels. Results are expressed relative to the dark levels of each genotype; data are means of biological triplicates +/- SD.

Figure 4: Reduced light-induced decline in phyA levels in *cop1-4* depends on the presence of metabolisable sugar in the growth media. Total protein extracts from 3-day-old etiolated Col-0, *cop1-4* and *spa1spa2spa3* seedlings transferred into red light (50µmol m^{-2} s^{-1}) for the indicated amount of time were separated on 8% SDS-PAGE gels, western blotted and probed with anti phyA and anti DET3 antibodies. (A, C, E, G) Representative western blots. (B, D, F) Quantification of phyA levels. Seedlings were either grown on ½ strength MS without sucrose (A, B, C and D), on ½ strength MS with 2% sucrose (E and F) or on ½ strength MS with 2% 3-O-CH_3-D-Glc (G). Results are expressed relative to the dark levels of each genotype; data are means of biological triplicates +/- SD.

Figure 5: The effect of sucrose in the growth media on light-regulated phyA abundance in *cul1* and *cul4*. Total protein extracts from 3-day-old etiolated Col-0, *cul1* and *cul4* seedlings growth on ½ strength MS with 2% sucrose transferred into red light (50µmol m^{-2} s^{-1}) for the indicated amount of time were separated on 8% SDS-PAGE gels, western blotted and probed with anti phyA and anti DET3 antibodies. (A, C) Representative western blots. (B, D) Quantification of phyA levels. Results are expressed relative to the dark levels of each genotype; data are means of biological triplicates +/- SD.

Figure 6: Effect of COP1, and CUL1 on *PHYA* transcript levels. Expression levels of *PHYA* in 3-day-old etiolated Col-0, *cop1-4* and *cul1* seedlings grown on ½ strength MS with or without 2% sucrose either kept in the dark or exposed to 4 hours of red light (50µmol m^{-2} s^{-1}) were analyzed by reverse transcription followed by real-time PCR. *EF1* and *YLS8* were used as house keeping genes. Data are normalized to *PHYA* in etiolated wild type and correspond to the mean +/- SD of three independent biological replicates with technical triplicates for each sample.

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

Supplemental information

Supplemental Figure S1: Cullin3 is dispensable for light-induced phyA degradation. Total protein extracts from 3-day-old etiolated Ws, *cul3a-1*, Col-0, *cul3a-2* and the viable *cul3a-3cul3b-1* seedlings transferred into red light (50µmol m^{-2} s^{-1}) for the indicated amount of time were separated on 8% SDS-PAGE gels, western blotted and probed with anti phyA and anti DET3 antibodies.

Supplemental Figure S2: phyA levels in etiolated seedlings. Total protein extracts from 3-day-old etiolated seedlings were separated on 8% SDS-PAGE gels, western blotted and probed with anti phyA and anti DET3 antibodies. Results are expressed relative to the dark levels of one of the Col-0 sample; data are means of biological triplicates +/- SD. (A) Col-0 and *cul1/axr6-3* grown on ½ strength MS without sucrose. (B) Col-0 and *cop1* grown on ½ strength MS with sucrose. (C) Col-0 and *spa1spa2spa3* grown on ½ strength MS without sucrose.

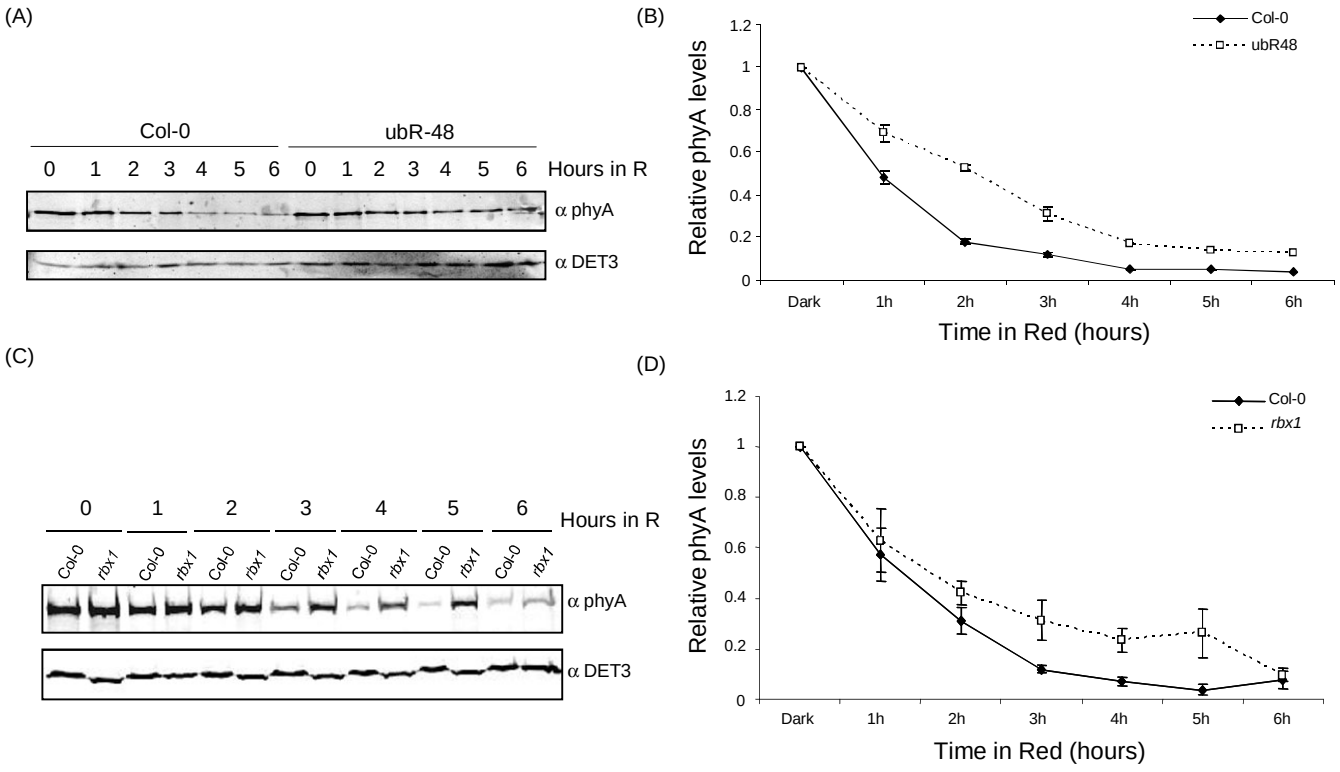
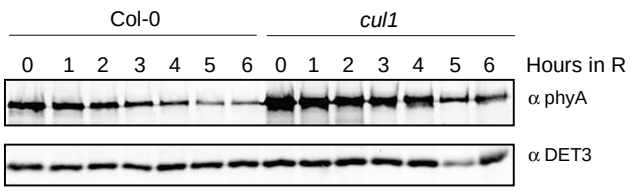


Figure 1: Cullin-based ubiquitin E3 ligases are required for the reduction of phyA levels in the light. Total protein extracts from 3-day-old etiolated Col-0, *RBX1* RNAi (*rbx1)* and ubR-48 expressing seedlings transferred into red light (50µmol m^{-2} s^{-1}) for the indicated amount of time were separated on 8% SDS-PAGE gels, western blotted and probed with anti phyA and anti DET3 antibodies. Seedlings were grown on ½ strength MS without sucrose. (A, C) Representative western blot. (B, D) Quantification of phyA levels. Results are expressed relative to the dark levels of each genotype; data are means of biological triplicates +/- SD.

(A)



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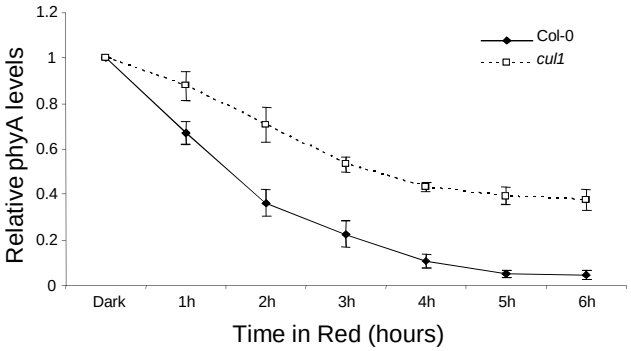

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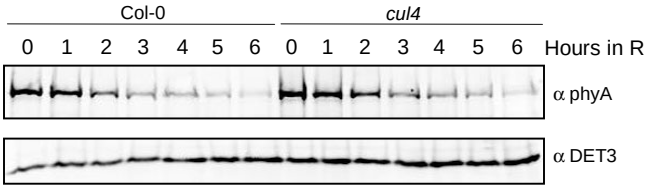
(A)



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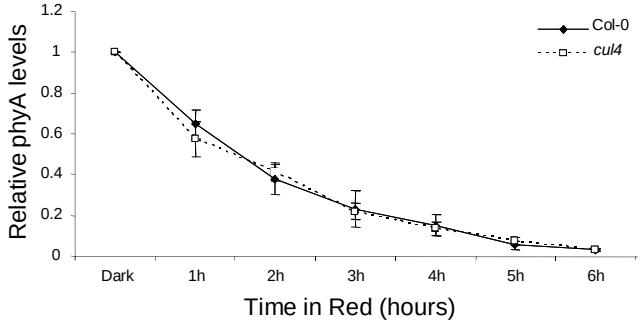

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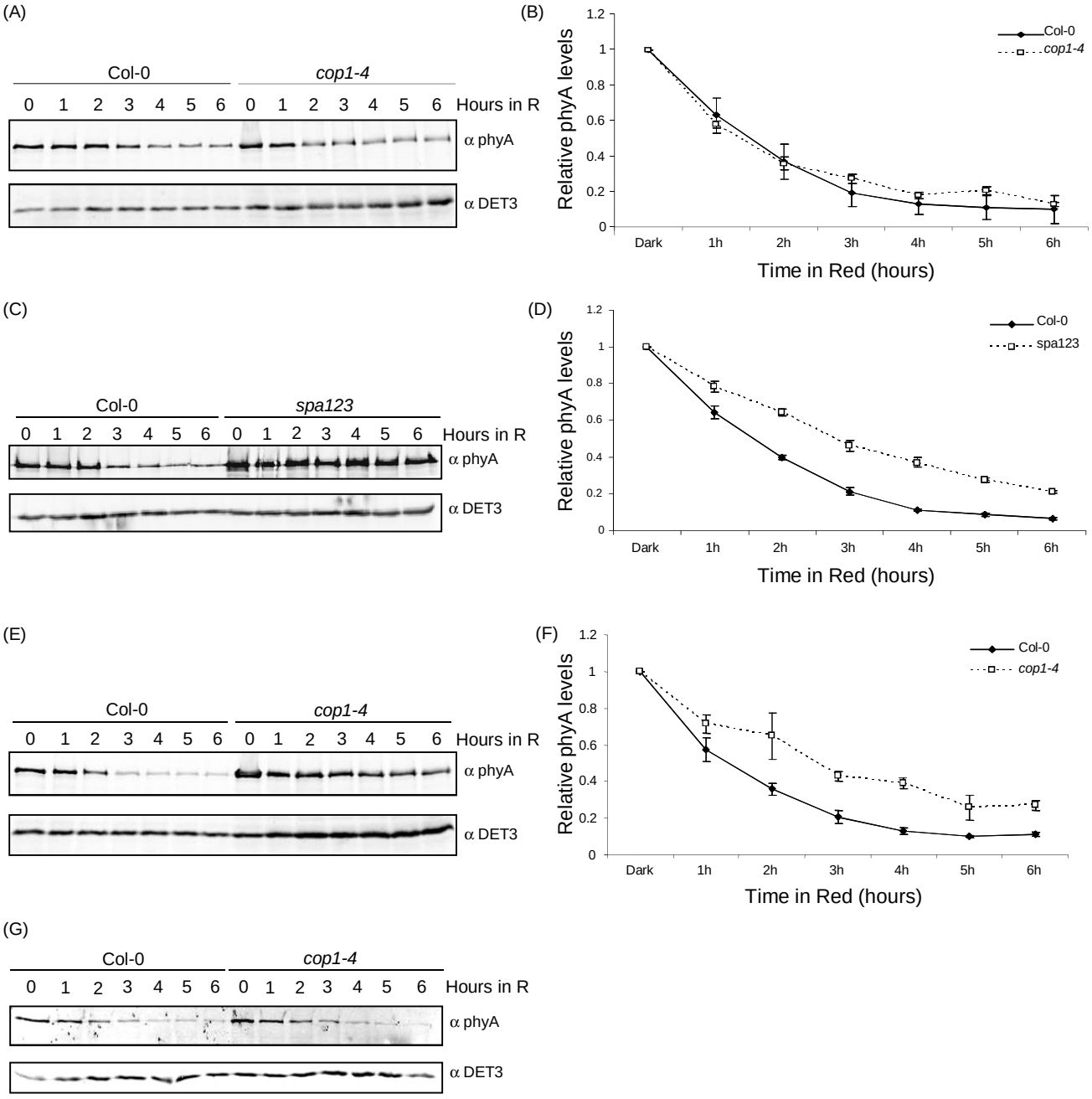

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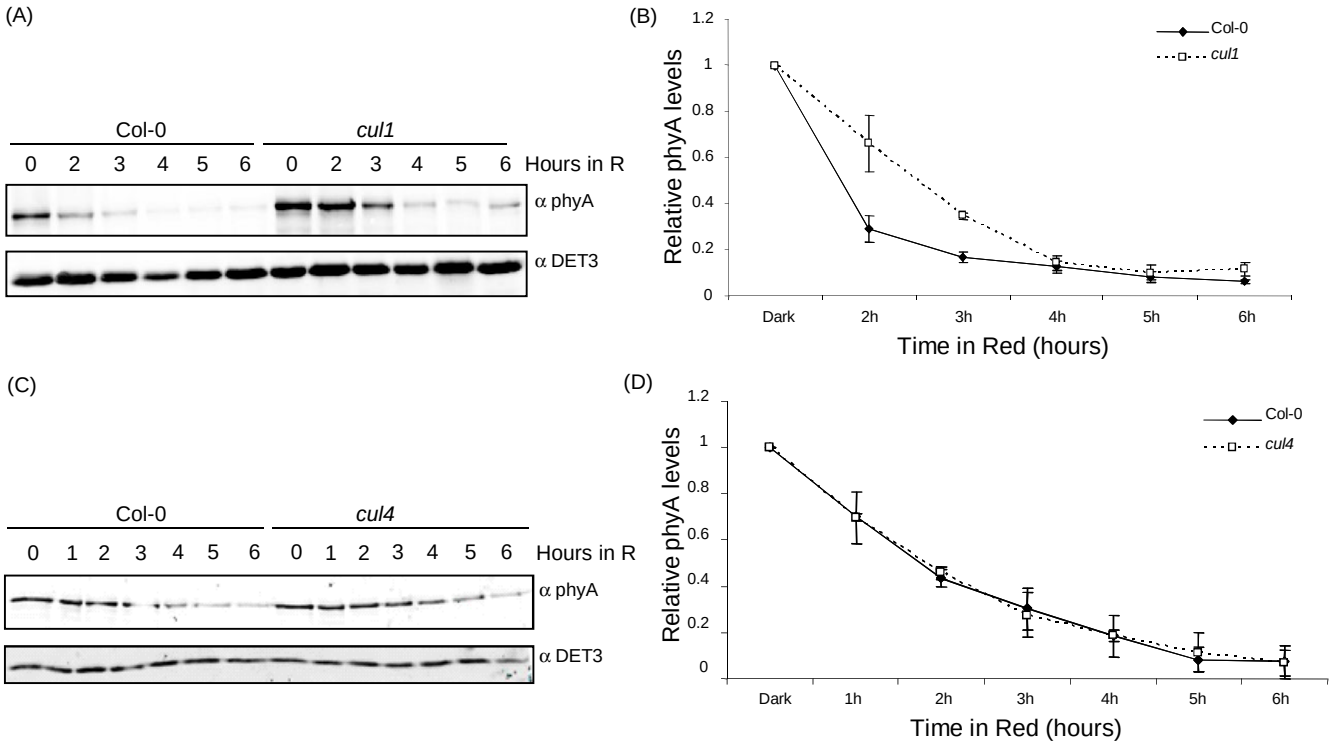

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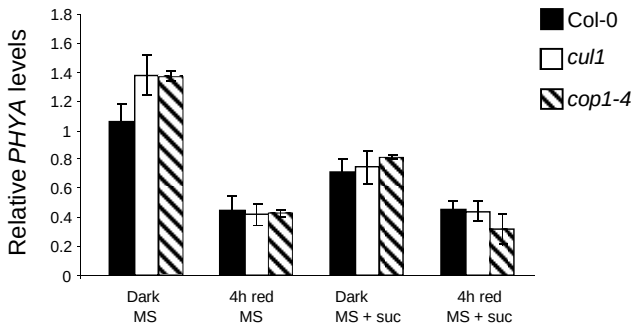

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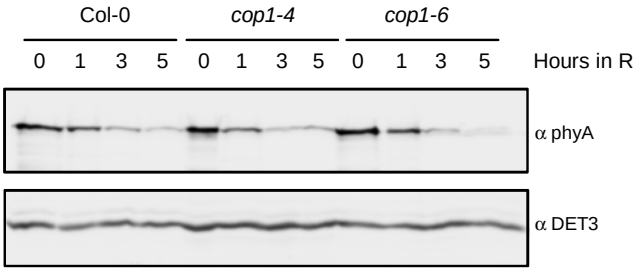

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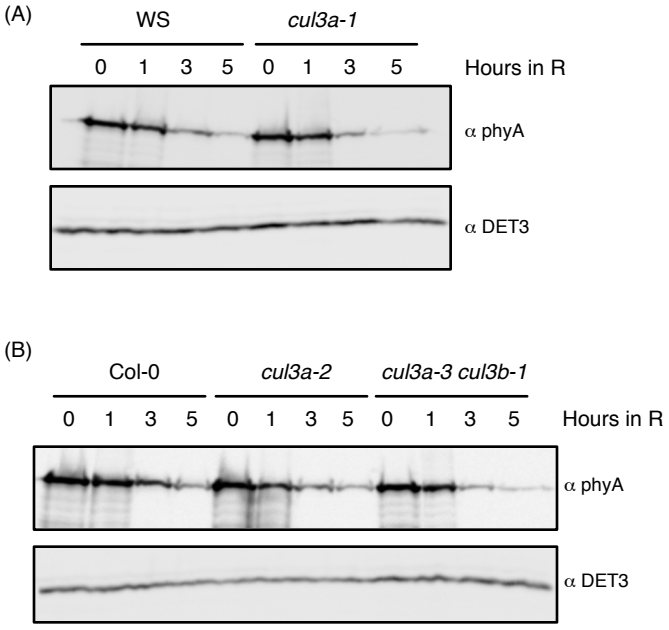

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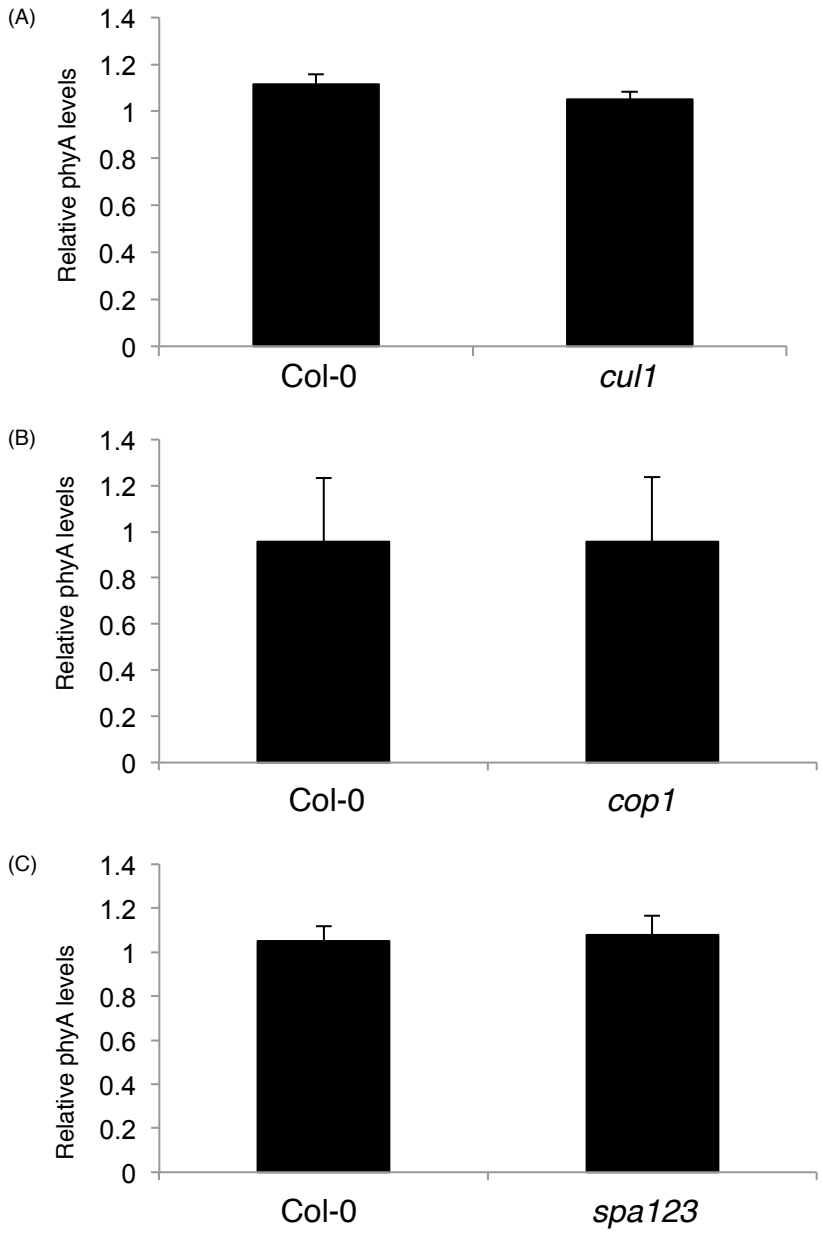

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