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Title page

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Title

Light-induced degradation of phyA is promoted by transfer of the photoreceptor into the nucleus.

Abstract

Higher plants possess multiple members of the phytochrome family of red, far-red light sensors to modulate plant growth and development according to competition from neighbors. The phytochrome family is composed of the light-labile phyA and several light-stable members (phyB-phyE in Arabidopsis). phyA accumulates to high levels in etiolated seedlings and is essential for young seedling establishment under a dense canopy. In photosynthetically active seedlings high levels of phyA counteract the shade avoidance response. phyA levels are maintained low in light-grown plants by a

combination of light-dependent repression of *PHYA* transcription and light-induced proteasome-mediated degradation of the activated photoreceptor. Light-activated phyA is transported from the cytoplasm where it resides in darkness to the nucleus where it is needed for most phytochrome-induced responses. Here we show that phyA is degraded by a proteasome-dependent mechanism both in the cytoplasm and the nucleus. However phyA degradation is significantly slower in the cytoplasm than in the nucleus. In the nucleus phyA is degraded in a proteasome-dependent mechanism even in its inactive Pr (red light absorbing) form, preventing the accumulation of high levels of nuclear phyA in darkness. Thus light-induced degradation of phyA is in part controlled by a light-regulated import into the nucleus where the turnover is faster. Although most phyA responses require nuclear phyA it might be useful to maintain phyA in the cytoplasm in its inactive form to allow accumulation of high levels of the light sensor in etiolated seedlings.

Keywords (4-6)

Phytochrome, phyA, proteasome, nuclear import, FHY1.

Introduction

The light environment provides important cues to autotrophic plants about time and place allowing them to tune their growth and development to the ever-changing light conditions. Plants measure light intensity, day length, spectral quality and light direction with UV-B photoreceptors, several families of blue light sensors and the phytochrome family of red/far-red receptors [5, 8, 21]. All higher plants possess two classes of phytochromes known as type I, which are light labile and type II, which are relatively light stable [8, 21]. Phytochrome A (phyA) is the only type I phytochrome in *Arabidopsis* while in this species phytochromes B, C, D and E (phyB-E) are light stable [8]. The phytochromes control seed germination, seedling de-etiolation, the timing of flowering and a suite of developmental responses known as the shade avoidance syndrome (SAS) [5, 8, 21].

Phytochromes are synthesized in their inactive red light absorbing form known as Pr [21, 24]. Upon light absorption they are converted to the active far-red absorbing form Pfr. Pfr can return to Pr either following absorption of far-red light or in a slow thermal reaction called dark reversion [21, 24]. Thus phytochromes typically initiate light responses in response to red light or in light environments leading to the formation of a relatively high ratio of activated phytochrome (Pfr/P_{tot}) [5, 21]. phyA has unique properties such as the ability to initiate light responses even if the light signal triggers a very low ratio of Pfr/P_{tot} [3, 5, 21, 24]. Such environments are encountered by an etiolated seedling emerging from the soil into a FR-rich environment indicative of vegetational shading [8].

In such environments phyA is the only photoreceptor mediating de-etiolation including inhibition of hypocotyl growth, unfolding and development of the cotyledons and setting up the photosynthetic apparatus [5, 8, 21]. The vital importance of phyA in young seedlings was demonstrated by genetic studies comparing wild type and *phyA* mutants developing under a dense canopy [34]. These unique features of phyA are presumably enabled by the high levels of this light sensor in etiolated seedlings [21, 24]. Moreover phyA is the only member of the phytochrome family that is efficiently transported into the nucleus in FR light [4]. However its exact mode of signal transduction in FR light is still debated [21, 24].

In photosynthetically active seedlings the levels of phyA drop considerably and phyA becomes significantly less abundant than phyB while phyA accounts for more than 80% of all phytochromes in etiolated seedlings [30]. The change of relative abundance of phyA and phyB has important developmental consequences in particular for the shade avoidance syndrome [8]. Direct sunlight has a ratio of red/far-red slightly above 1. Under a plant canopy this ratio drops significantly below 1 due to the strong absorption of red light by photosynthetic pigments while far-red light is readily transmitted. This change in light quality sensed by the phytochromes is used by shade-avoiding plants to induce the shade avoidance syndrome [8]. The shade avoidance syndrome includes the promotion of elongation growth responses in stems and petioles, a change in leaf angle leading to a more erect position and induction of reproductive growth [8]. By inhibiting elongation growth in low R/FR phyA counteracts the shade avoidance response. It has thus been proposed that the light-induced degradation of phyA constitutes an important mechanism

enabling de-etiolated plants to effectively compete for light in shaded environments [8, 18]. The strongly attenuated shade avoidance response in phyA over-expressing plants is consistent with this notion [1, 23].

The molecular mechanisms leading to a drastic reduction of phyA levels in photosynthetically active plants have only been partially elucidated. *PHYA* transcript levels are down regulated in response to light, however this effect is stronger in monocots than in dicotyledonous plants [2]. In Arabidopsis it has been shown that this down-regulation depends on both phyB and phyA [2]. Light-activated phyA from numerous species is ubiquitylated and targeted to the proteasome largely contributing to the rapid drop in phyA levels in the light [14, 29]. This primarily depends on sequences in the photosensory domain of phyA [6, 32, 33]. In Arabidopsis pharmacological experiments have shown that inhibition of the proteasome leads to a slower degradation of phyA in the light [28]. The ubiquitin E3 ligase COstitutively Photomorphogenic 1 (COP1) is involved in the light-induced degradation of phyA [28]. Moreover in a cullin 1 (*cull1*) loss-of-function mutant phyA is also more stable in the light [22]. Interestingly COP1 has been proposed to be part of cullin 4 based ubiquitin E3 ligase complex possibly suggesting that several mechanisms contribute to the degradation of phyA in the light (CUL1 and CUL4 based) [35].

Phytochromes reside in the cytoplasm in their inactive Pr form. Upon light activation they rapidly accumulate in the nucleus where they are required for most phytochrome-mediated responses [11, 13, 19, 25]. Different phytochromes utilize distinct mechanisms

to mediate regulated nuclear import [4, 7]. Light activation of phyB triggers a conformational change exposing an NLS thus leading to specific import of Pfr [4]. phyA relies on a more complex mechanism including the related proteins Far-red Hypocotyl 1 and FHY1-Like (FHY1 and FHL). These proteins possess a phyA interaction domain, an NLS and an NES. They specifically interact with the light-activated phyA in vitro and mediate nuclear import of the active photoreceptor [9, 12, 20, 25]. In addition light-induced accumulation of phyA relies on Far-red Hypocotyl 3 and FAR-red impaired response 1 (FHY3 and FAR1) a pair of transposase-related transcriptional activators needed for the expression of *FHY1* and *FHL* [16]. Light also triggers the formation of phyA speckles both in the cytoplasm and the nucleus [4]. It has been proposed that these structures represent sites of phyA turnover suggesting that degradation of the light-activated photoreceptor occurs both in the cytoplasm and the nucleus [15, 17, 28].

In order to test this idea further we have studied changes in phyA levels in response to light both in the nucleus and in the cytoplasm. We compared the wild type with seedlings in which phyA remains in the cytoplasm and seedlings in which phyA is constitutively nuclear. Our data indicate that phyA degradation is proteasome-mediated in both subcellular compartments. Interestingly phyA degradation is more effective in the nucleus than in the cytoplasm and proteasome-mediated degradation of the inactive Pr form of phyA was only observed in the nucleus. Thus the light-induced reduction in phyA levels is in part an indirect consequence of nuclear import of the light-activated photoreceptor. Our data suggest that maintaining inactive phyA in the cytoplasm allows a

higher accumulation of this photoreceptor in the dark, which might be important for the early light responses critically depending on phyA.

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 35mm² x 20mm). The plates were stored at 4°C in the dark during 3 days for stratification followed by a 6 hour white light (100µmol m⁻²s⁻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 or Far-red light from LED sources with λ_{max} at 670 and 730nm respectively). The following genotypes were used in this study. Col was used as a wild type. *fhy1fhl*, *phyA-211* and *phyA-211* expressing either *PHYA_{pro}:PHYA-GFP* or *PHYA_{pro}:PHYA-NLS-GFP* were described previously [9, 25].

Quantitative western blot analysis (Li-Cor)

Quantitative western blots were performed essentially as described in [32]. Fifty seedlings by genotype were exposed either to continuous red (50 µmol m⁻² s⁻¹) or to continuous far-red (5 µmol m⁻² s⁻¹) light, and 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 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) [31] or a rabbit polyclonal antibody against DET3 [27] 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. We use DET3 as a loading control because our experiments have shown that DET3 protein levels are remarkably stable in young seedlings (in a variety of conditions). We reached this conclusion by carefully quantifying total protein levels of different extracts subjected to western blot analysis and also by comparing DET3 signal with the signal given by commercially available antibodies used by others as a loading control. Analysis of publicly available micro-array data also shows that *DET3* RNA levels remain constant upon light perception in etiolated seedlings. To determine the apparent half-lives of phyA we used the data presented in Figures 1B, 3B and Suppl. 1B. The log₂ of the relative phyA levels were expressed according to time in 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ red light and linear regressions were calculated. The regression coefficient of the different experiments varied between 0.94 and 0.99 demonstrating that these functions are a very good representation of our data. The apparent half-lives of phyA was then calculated using these functions.

Proteasome inhibitor treatment.

Seedlings were incubated in liquid ½ strength MS medium containing 50µM MG132 (Calbiochem) for 2 hours (equivalent DMSO concentration for the control). Seedlings were then transferred into red light for increasing amounts of time and total proteins were extracted as described above.

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).

Confocal microscopy

Three-day-old etiolated seedlings of transgenic lines expressing either phyA-GFP or gphyA-NLS-GFP [9] were grown as described above. Seedlings were mounted in water between glass slides separated with two layers of transparent tape and sealed using nail polish. Samples were visualized using an inverted confocal laser scanning Carl Zeiss LSM 510 Axiovert 200M Zeiss microscope with a standard filter set. Laser monochromatic excitation light $\lambda_{exc}=488\text{nm}$ was obtained from Argon/Krypton gas mixture. Emission light was collected using a short-pass 505-530nm filter for GFP signal (converted into green) and long-pass 650nm filter for plastid signal (converted into red). Image analysis was done with the Zeiss LSM software. The same analysis was done after 1 minute of red irradiation with a monochromatic laser ($\lambda=660\text{nm}$) followed by 4 minutes in darkness.

Results

phyA enters the nucleus upon light activation. Light-induced changes in phyA abundance may thus be due to a combination of several levels of regulation, including repression of *PHYA* transcript levels and ubiquitin-mediated degradation of phyA both in the nucleus and the cytoplasm [2, 14, 28]. In order to verify whether the light-induced change in phyA subcellular location modulates the light-induced decline in phyA levels we analyzed phyA levels in the *fhy1fhl* double mutant, a genetic background in which phyA nuclear import is prevented [9, 12, 25]. Wild type (Col) and *fhy1fhl* etiolated seedlings were transferred either into red or far-red light and phyA accumulation was determined by quantitative western blot analysis (Figure 1). Upon transfer into red light phyA levels dropped very quickly in the wild type with about 20 % of the dark levels remaining after 4 hours in the light (Figure 1 A, B). This red-light induced decline was significantly slower in *fhy1fhl* where after 4 hours those seedlings still had about 40% of the phyA levels in darkness (Figure 1 A, B). This data was used to calculate the apparent half-lives of phyA under these growth conditions and we found that the phyA half-life in *fhy1fhl* was the double from the wild-type value (about 3 hours compared to 1 hour and 30 minutes in Col) (Table 1). Similar results were obtained when the seedlings were transferred into far-red light but the kinetics of phyA light-induced decline was much slower which is consistent with the small ratio of Pfr/Ptot triggered by far-red light. Again the light induced reduction in phyA levels was significantly slower in the *fhy1fhl* double mutant (Figure 1 C, D).

One possible interpretation of this result is that light-induced phyA degradation is slower in the cytoplasm than in the nucleus. In order to test this we compared plants expressing phyA-GFP with plants expressing phyA-NLS-GFP in which phyA is constitutively nuclear [9]. Using confocal microscopy we confirmed that in the hypocotyls of etiolated seedlings phyA-GFP was found in the cytoplasm while phyA-NLS-GFP was exclusively nuclear (Figure 2). phyA-GFP transport into the nucleus of etiolated seedlings was triggered by 1 minute illumination with the red laser from the microscope followed by 4 minutes of darkness. This experiment showed that nuclear accumulation in response to red light was very fast (Figure 2). Moreover this experiment confirmed that nuclear body (or speckle) formation was specifically induced by red light (Figure 2). phyA levels were then compared in those two lines by quantitative western blot analysis using etiolated seedlings transferred into red light. This experiment showed that phyA-GFP was rapidly degraded in both lines. The decline in phyA levels was slightly faster for the constitutively nuclear phyA-NLS-GFP than for phyA-GFP (Figure 3). In order to confirm the more rapid degradation of phyA when it is constitutively nuclear we compared the degradation of endogenous phyA with the degradation of phyA-NLS expressing plants (in the *phyA* background) [9]. This experiment confirmed that when constitutively present in the nucleus phyA is turned over more rapidly (Supplementary Figure 1) (table 1). The modest difference in degradation kinetics between the photoreceptor with light-regulated nuclear import and a constitutively nuclear phyA might be the consequence of the very rapid nuclear import observed for phyA-GFP in response to red light (Figure 2).

Light does not only lead to phyA degradation but also to a decline in *PHYA* transcript levels [2]. Although this transcriptional regulation of *PHYA* is less important in dicotyledonous plants than in monocots [2], we analyzed *PHYA* transcript abundance in the different genotypes by quantitative RT-PCR. In etiolated seedlings *PHYA* levels were very similar in Col, *phyA* expressing phyA-GFP and *fhy1fhl* (Figure 4). In all cases a red light treatment led to a comparable decline in *PHYA* abundance (Figure 4). Interestingly the phyA-NLS-GFP line had much higher levels of *PHYA* than all other genotypes. However the light-induced decline of *PHYA* was normal in this genotype (Figure 4). The situation at the RNA level contrasted with the abundance of phyA-NLS-GFP. Despite having almost twice as much RNA those etiolated seedlings expressed less than half the phyA protein level than all other genotypes (Figure 4). These results indicate that the slower decline of phyA in *fhy1fhl* was not due to an altered light-regulated *PHYA* abundance. Moreover they show that when phyA is constitutively present in the nucleus the protein accumulates to lower levels than in the cytoplasm even in its inactive Pr form.

phyA degradation follows ubiquitylation and is proteasome mediated [14, 28]. We thus decided to verify whether proteasome-mediated degradation of phyA occurs both in the nucleus and the cytoplasm by comparing the effect of the proteasome inhibitor MG132 on the decline of phyA levels in *fhy1fhl* (cytoplasmic phyA) and phyA-NLS-GFP (nuclear phyA). MG132 effectively slowed down the reduction in phyA levels in both genotypes indicating that phyA degradation is mediated by the proteasome in both compartments (Figure 5). The effect of MG132 was more obvious in phyA-NLS-GFP plants than in *fhy1fhl* presumably because in phyA-NLS-GFP the decline in phyA was

faster (Figure 5). Interestingly MG132 significantly increased the level of phyA-NLS-GFP in etiolated seedlings while it did not have an effect on phyA in etiolated *phy1fhl* (Figure 5). To verify that this effect was not an artifact due to the presence of GFP in the phyA-NLS-GFP construct we compared the effect of MG132 on etiolated seedlings expressing phyA-GFP with seedlings expressing phyA-NLS-GFP. This experiment showed that MG132 had no effect on the accumulation of the cytosolic phyA-GFP while it led to a higher accumulation of the nuclear phyA-NLS-GFP protein (Supplementary Figure 2, Figure 2). This data suggests that when present in the nucleus phyA in the Pr conformation is turned over more rapidly than in the cytoplasm and that this degradation is proteasome dependent (Figure 5, Supplementary Figure 2).

Discussion

phyA has a number of properties that are unique among all phytochromes. Those include the capacity of initiating light responses such as the promotion of seed germination and de-etiolation in light conditions that trigger only a very minimal activation of the photoreceptor (very low ratio of Pfr/Ptot) [5, 8, 21]. In addition phyA levels decline rapidly upon light activation a property which is important to prevent phyA from antagonizing the shade avoidance syndrome in photosynthetically active plants [1, 23]. The light-labile nature of phyA is thus an important property of this photoreceptor, which is still relatively poorly understood.

Degradation of phyA correlates with phototransformation of Pr into Pfr. This primary light reaction also triggers import of phyA into the nucleus upon conformer-specific interaction with FHY1 and FHL [9, 12, 25]. We thus decided to investigate whether the light-induced degradation of phyA is also due to a change in subcellular localization. By comparing the light-induced reduction of phyA levels in Col and *fhy1fhl* we showed that in this double mutant phyA remains much more stable in the light (Figure 1). We do not have an explanation for why this effect was not observed in a previous study [25], however we would like to point out that these authors did not perform quantitative western blots. Importantly the reduced decline in phyA levels is not due to an effect of *fhy1fhl* on the light-induced reduction of *PHYA* transcript levels (Figure 4). One possible interpretation of this result which has previously been proposed by others is that FHY1 and FHL are required to degrade light activated phyA [26]. This possibility is unlikely

given that the stability of phyA-NLS-GFP is not altered in the *fhy1* mutant background [9]. In other words when phyA is constitutively nuclear FHY1 does not affect the stability of the photoreceptor. Formally it is thus still possible that FHY1 and FHL are required for the rapid degradation of light-activated cytoplasmic phyA. Nevertheless taken collectively our data are more consistent with the notion that light-activated phyA is more stable in *fhy1fhl* because the protein remains in the cytoplasm. Indeed when phyA is constitutively nuclear such as in plants expressing phyA-NLS-GFP, phyA accumulates to relatively low levels in etiolated seedlings despite the presence of high mRNA levels (Figures 3 and 4).

In plants expressing the constitutively nuclear phyA-NLS-GFP construct this protein never accumulates to very high levels despite the presence of more *PHYA-NLS-GFP* transcript than in the wild type or the phyA-GFP control lines (Figure 4). Moreover phyA-NLS-GFP levels can be increased when treating etiolated seedlings with the proteasome inhibitor MG132 while this drug had no effect in dark-grown control lines (Figure 5, supplementary Figure 2). This result suggests that when present in the nucleus phyA might be less stable in the Pr conformation than when present in the cytoplasm. This is consistent with the observation that in vitro COP1 interacts with phyA both in the Pr and the Pfr conformation [28]. Thus maintaining phyA in the cytoplasm in dark-grown seedlings may contribute to the very high accumulation of the photoreceptor observed in etiolated plantlets. Maintaining high levels of phyA in etiolated seedlings may be important for the seedling emerging from the soil to detect the very first signs of light.

Moreover cytoplasmic accumulation of phyA is presumably required for a number of cytosolic phytochrome responses [25].

When transferred into the light proteasome inhibitors slowed down the degradation of phyA both in the cytoplasm (in *fhy1fhl*) and in the nucleus (in phyA-NLS-GFP expressing plants), thus suggesting that proteasome mediated degradation occurs in both cellular compartments. As discussed above a high degradation rate for phyA in light grown plants is important to prevent the inhibition of the shade avoidance syndrome. Moreover *in vivo* dark-reversion is undetectable for phyA [10], thus one effective way to prevent excessive signaling from the light activated phyA is to degrade the protein. Our results suggest that one element of the light-regulated stability of phyA is the light-induced nuclear import of the photoreceptor. It will be interesting to determine whether different degradation mechanisms control phyA stability in the nucleus and the cytoplasm. This is a distinct possibility given that COP1 and CUL1 which presumably belong to distinct ubiquitin E3 ligase complexes have both been implicated in the control of phyA abundance [22, 28].

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Figure Legends

Figure 1: The light-induced reduction of phyA levels is slower in *fhy1fhl*. Total protein extracts from 3-day-old etiolated seedlings of Col-0 and *fhy1fhl* transferred for increasing amounts of time into red light ($50\mu\text{mol m}^{-2} \text{s}^{-1}$) or far-red light ($5\mu\text{mol m}^{-2} \text{s}^{-1}$) were separated on 8% SDS-PAGE gels, blotted and probed with anti phyA and anti DET3 antibodies. (A & C) Representative western blots from the red and far-red experiments respectively. (B & D) quantification of phyA levels in red and far-red following the method of [32]. Results are expressed relative to the dark levels of each genotype (set to 1); data are means of biological triplicates +/- SD.

Figure 2: phyA-NLS-GFP is constitutively localized in the nucleus and form speckles after red light irradiation. 3-day-old etiolated seedlings of transgenics *phyA* null mutants expressing either phyA-GFP (A and C) or phyA-NLS-GFP (B and D) were analyzed by confocal microscopy using an immersion objective 40X. Transgenics lines were kept in the dark (upper panel) or irradiated with a red laser (λ 660nm) for 1 minute followed by 4 minutes in darkness prior to microscopic examination (lower panel). (C) White arrows indicate the nuclei of cells expressing a phyA-GFP fusion. (D) The inset shows a zoom (factor 6.6) of one nucleus. Confocal microscopy was carried out using an inverted confocal LSM510 Axiovert 200M Zeiss microscope. The scale bars represent 20 μm .

Figure 3: Constitutively nuclear phyA-NLS-GFP levels decline rapidly in response to light. Total protein extracts from 3-day-old etiolated seedlings of *phyA-GFP* and *phyA-*

NLS-GFP expressing seedlings shifted for increasing amounts of time into red light ($50\mu\text{mol m}^{-2} \text{s}^{-1}$) were separated on 8% SDS-PAGE gels, blotted and detected with anti phyA and anti DET3 antibodies. (A) A representative western blot. (B) Quantification of phyA levels following the method of [32]. Results are relative to the dark levels of each genotype. Data are means of biological triplicates +/- SD.

Figure 4: the phyA levels observed in *fhy1fhl* are not due to an altered light-induced decline of *PHYA* mRNA. (A) Expression levels of *PHYA* in 3-day-old etiolated seedlings of Col-0, *fhy1fhl*, phyA-GFP and phyA-NLS-GFP kept either in the dark or exposed to 4 hours of red light ($50\mu\text{mol m}^{-2} \text{s}^{-1}$) were analyzed by reverse transcription followed by real-time PCR. *EF1* and *YLS8* were used as house keeping genes. Data correspond to the mean +/- SD of three independent biological replicates with technical triplicates for each sample. (B) Quantification of phyA levels from 3-day-old etiolated seedlings of Col-0, *fhy1fhl*, phyA-GFP and phyA-NLS-GFP kept in the dark or exposed to 4 hours of red light ($50\mu\text{mol m}^{-2} \text{s}^{-1}$) following the method of [32]. Results are relative to the dark level of Col-0, data are mean of biological triplicates +/- SD.

Figure 5: phyA degradation in the nucleus and in the cytosol is delayed by inhibitors of the proteasome. Total protein extracts from 3-day-old etiolated seedlings of *phyA-NLS-GFP* or *fhy1fhl* incubated or not with the proteasome inhibitors MG132 (MG132) and transferred for increasing amounts of time into red light ($50\mu\text{mol m}^{-2} \text{s}^{-1}$) were separated on 8% SDS-PAGE, blotted and probed with anti phyA and anti DET3 antibodies. (A & C) Representative western blots of *phyA-NLS-GFP* and *fhy1fhl* respectively. (B & D)

quantifications of phyA levels in *phyA-NLS-GFP* and *fhy1fhl* following the method of [32]. Results are relative to the dark levels of each genotype, except for the insets where they are represented relative to the –MG132 level. Data are means of biological triplicates +/- SD.

Table 1: Apparent half lives of phyA in 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ red light. The apparent phyA half-lives were calculated using data presented on Figures 1B, 3B and Supplementary Figure 1B (see materials and methods). It is important to point out that this data was obtained in seedlings that were not treated with translation inhibitors and therefore do not represent the actual half-life of the protein.

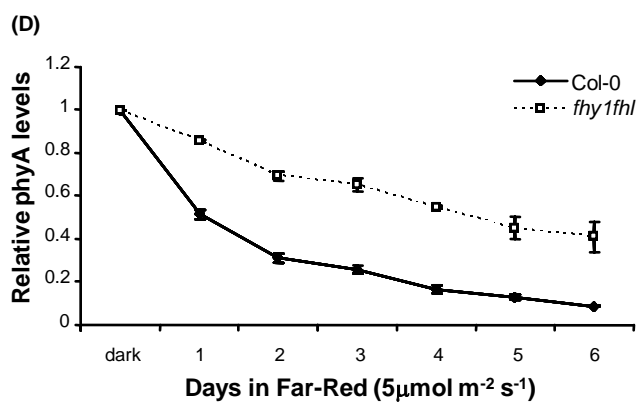
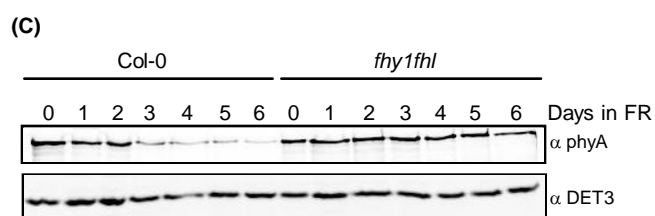
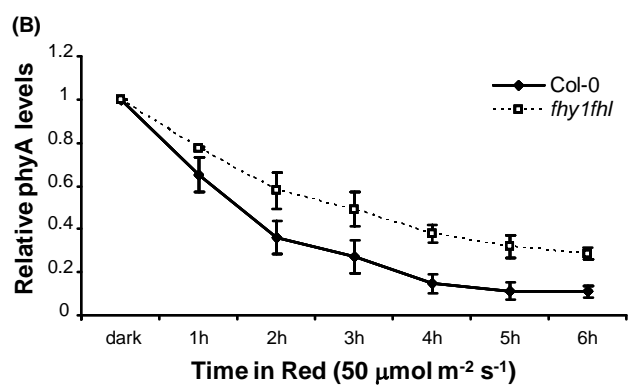
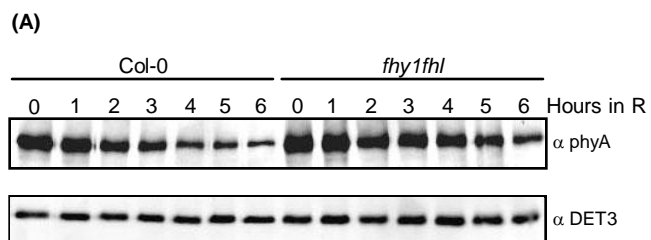


Figure 1

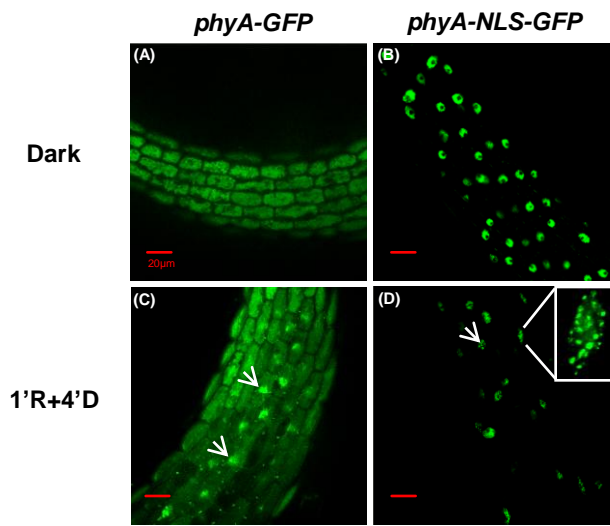
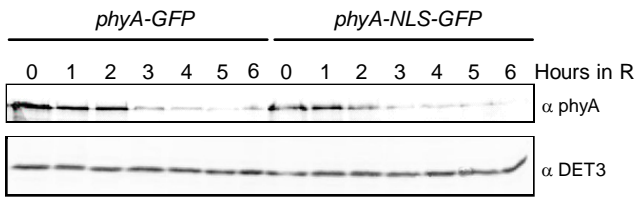


Figure 2

(A)



(B)

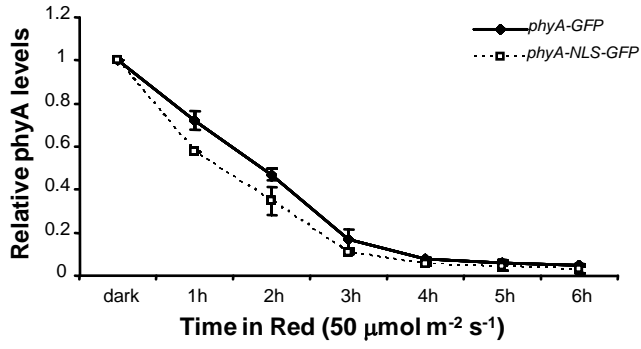


Figure 3

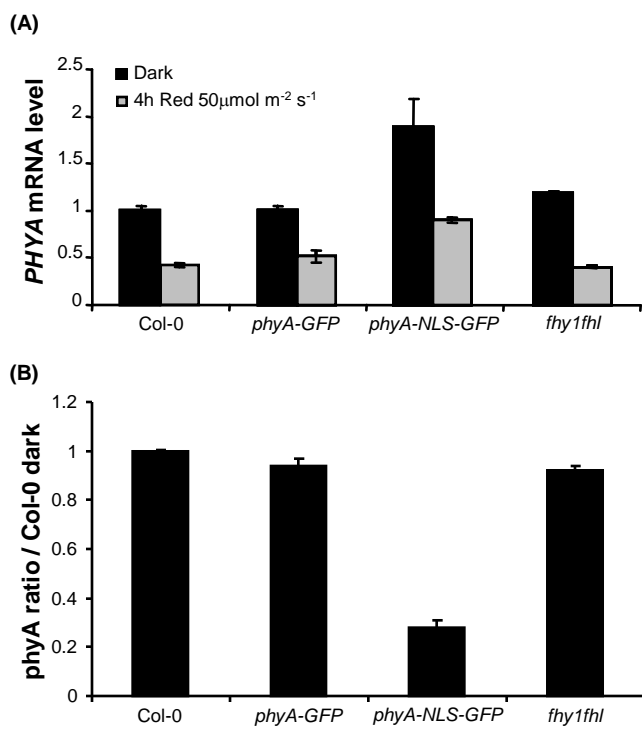


Figure 4

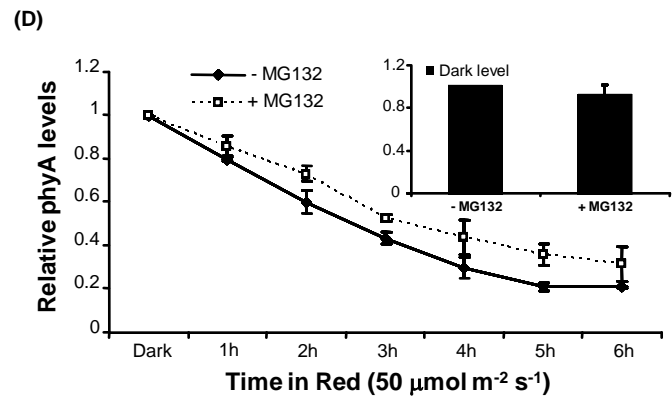
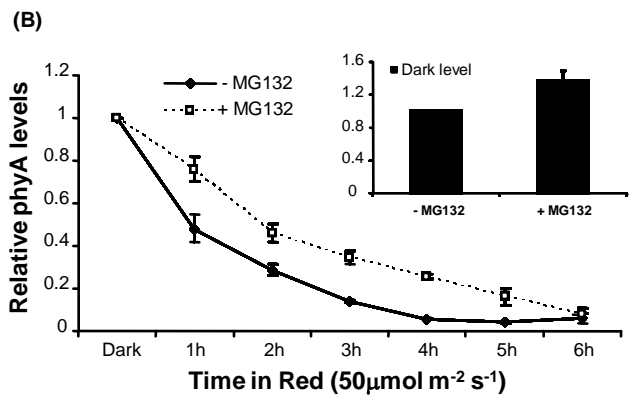
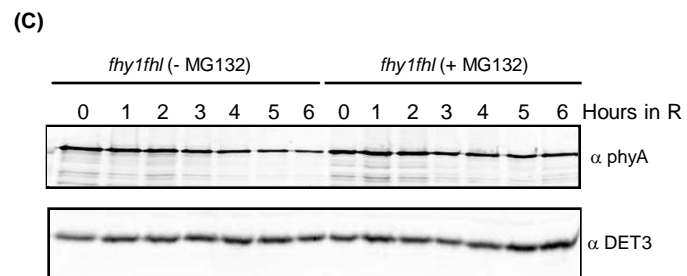
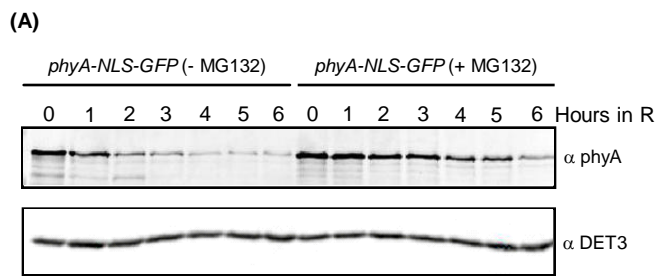


Figure 5

Genotype	Half-life of phyA
Col	89 minutes
<i>phy1fhl</i>	178 minutes
phyA-GFP	84 minutes
phyA-NLS-GFP	66 minutes
phyA-NLS	44 minutes

Table 1

Supplementary material

Figure legends

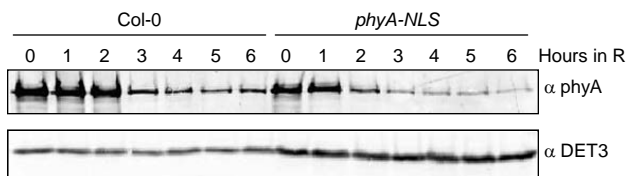
Supplementary figure 1: phyA-NLS levels decline faster in response to light than endogenous phyA. Total protein extracts from 3-day-old etiolated seedlings of Col-0 and *phyA-NLS* seedlings [1] shifted for increasing amounts of time into red light ($50\mu\text{mol m}^{-2} \text{s}^{-1}$) were separated on 8% SDS-PAGE gels, blotted and detected with anti phyA or anti DET3 antibodies. (A) A representative western blot. (B) Quantification of phyA levels following the method of [2]. Results are relative to the dark levels of each genotype, data are means of biological triplicates +/- SD.

Supplementary figure 2: the degradation of phyA in the nucleus is independent of its conformation (Pr or Pfr). Total protein extracts from 3-day-old etiolated seedlings of *phyA-NLS-GFP* or *phyA-GFP*, incubated or not with the proteasome inhibitors MG132 (MG132) for 2 hours in the dark were separated on 8% SDS-PAGE, western blotted and probed with anti phyA or anti DET3 antibodies. (A) Representative western blots of *phyA-NLS-GFP* and *phyA-GFP* respectively. (B) Quantifications of phyA levels relative to DET3 in *phyA-NLS-GFP* and *phyA-GFP* following the method of [2]. Data are means of technical triplicates +/- SD.

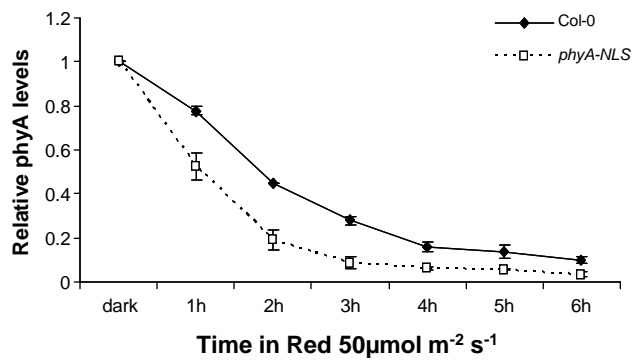
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(A)

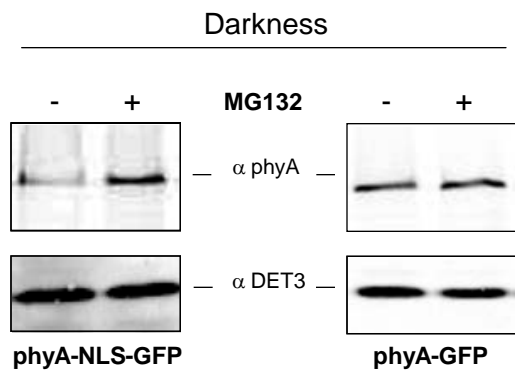


(B)

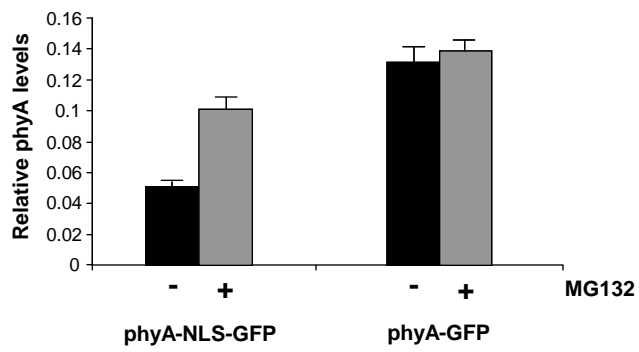


Supplementary Figure 1

(A)



(B)



Supplementary Figure 2