MOLECULAR MECHANISMS OF PHYTOCHROME A NUCLEAR TRANSPORT AND DOWNSTREAM SIGNALING

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This dissertation is dedicated
to my husband, Bernhard, and my children.

Thank you for being here,
for your love and support

"EVERY ACCOMPLISHMENT STARTS WITH THE DECISION TO TRY"

(Author unknown)

TABLE OF CONTENTS

Table of contents I
Abbreviations II
List of figures IV
Summary V
Zusammenfassung VI
1 Introduction 1
1.1 Phytochromes function as red/far-red photoreceptors 2
1.2 Modes of action of phytochromes 5
1.3 Phytochromes light-regulated subcellular localization 6
1.4 The phytochromes signaling pathway 7
1.5 Ecological function and evolution of phytochromes 11
1.6 Ojective of this work 16
2 Results and discussion 17
2.1 Requirement of FHY1/FHL for nuclear transport of PHYA 17
2.2 Function of FHY1/FHL in phyA nuclear downstream signaling 22
2.3 Possible cytosolic function of phyA 24
2.4 Evolutionary aspects of FHY1-dependent phyA nuclear transport 26
2.5 Repression of the COP1/SPA complex is directly mediated by phytochromes 27
2.6 Multiple mechanisms inhibit the COP1/SPA complex 30
2.6.1 Nuclear/cytoplasmic translocation of COP1 30
2.6.2 Phytochrome-mediated SPA proteins turnover 34
2.7 Roles of SPA proteins in other light-regulated processes 35
2.8 SPAs might be required to achieve wavelength specificity in visible light 37
References 40
List of publications and manuscripts 50
Curriculum Vitae 133
Acknowledgements 134

ABBREVIATIONS

°C	degree(s) Celsius
ABA	abscisic acid
ABI5	ABA INSENSITIVE 5
APA	active phytochrome A binding
APB	phytochrome B-binding
B	blue light
bHLH.	basic helix–loop–helix
CHS	chalcone synthase
CO	CONSTANS
COP1	CONSTITUTIVELY PHOTOMORPHOGENIC1
CRY	cryptochrome
CSN1	CONSTITUTIVELY NUCLEAR LOCALIZED SUBUNIT 1
CUL4	CULLIN 4
DDB1	DAMAGED DNA BINDING 1
e.g.	exempli gratia (for example)
et. al	et alii (and others)
FHL	FHY1-LIKE
FHY1	FAR-RED ELONGATED HYPOCOTYL1
FKF	FLAVIN-BINDING KELCH REPEAT F-BOX
FLIM	fluorescence lifetime imaging
FR	far-red light
FRET	Förster resonance energy transfer
FT	FLOWERING LOCUS T
GAF	cGMP phosphodiesterase/adenylyl cyclase/FhlA
GFP	green fluorescent protein
GUS	β-glucuronidase
HFR1	LONG HYPOCOTYL IN FAR-RED 1
HIR	high-irradiance response
HKRD	histidine kinase-related domain
HY5	ELONGATED HYPOCOTYL 5
i.e.	id est (that is)
LAF1	LONG AFTER FAR-RED LIGHT 1
LFR	low-fluence response
LKP2	LOV KELCH REPEAT PROTEIN 2
LUC	luciferase
mCherry	monomeric cherry fluorescent protein
NB	nuclear bodies

NES	nuclear export signal
NLS	nuclear localization sequence
OPM	output module
PAS	Period/Arnt/Single-Minded
Pfr	far-red-absorbing form of phytochrome (active)
PHOT	phototropin
Phy	Phytochrome (Holoprotein with chromophore)
PIF	PHYTOCHROME INTERACTING FACTOR
PKS	PHYTOCHROME KINASE SUBSTRATE
Pr	red-absorbing form of phytochrome (inactive)
PRD	PAS-related domain
PRR9	PSEUDO-RESPONSE REGULATOR 9
PSM	photo-sensing module
R	red light
RBX1	RING BOX 1
SAP	sequestered areas of phytochrome
SAR	shade avoidance response
SPA	SUPPRESSOR OF phyA-105
TF	transcription factor
UV-B	ultra-violet B
UVR8	UV RESISTANCE LOCUS 8
VLFR	very-low-fluence response
W	white light
Y2H	yeast two-hybrid
YFP	yellow fluorescent protein
ZTL	ZEITLUPE

LIST OF FIGURES

1 Introduction:

Figure 1.1: Domain structure of canonical plant phytochromes............................. 3

Figure 1.2: Absorption spectra of the inactive (Pr) and active (Pfr) forms of phytochrome and wavelength-dependent proportion of phytochrome in the Pfr form. 4

Figure 1.3: Simplified model of the phyA light signaling pathways. 9

Figure 1.4: Growth and developmental processes regulated by different photoreceptors. 12

2 Results and Discussion

Figure 2.1: PhyA nuclear translocation depends on two functional homologs FHY1 and FHL. 20

Figure 2.2: Molecular network of the shade avoidance response. 32

Figure 2.3: Overlapping and distinct functions of SPA proteins during plant development.. 38

SUMMARY

The red/far-red photoreceptors of the phytochrome family are important for the regulation of a multitude of responses in plants, including germination, growth and adaptation to environmental changes. Among the five members of phytochrome present in *Arabidopsis thaliana*, phytochrome A and B (phyA and phyB) play a dominant role. The light-mediated nuclear translocation of photoactivated phyA is one of the key steps in far-red light signaling and requires the interaction with the two functional homologs FAR-RED ELONGATED HYPOCOTYL1 (FHY1) and FHY1-LIKE (FHL). Besides their function as phyA transport proteins, FHY1 and FHL have been attributed a role in mediating the assembly of phyA signaling complexes in the nucleus. The mutant analyses in Arabidopsis here reported provides insight into the role of FHY1/FHL in phyA downstream signaling in the nucleus. The main resulting conclusions are that FHY1 and FHL are (i) not required for far-red light nuclear signaling, and (ii) that FHY1/FHL-mediated light-dependent phyA nuclear transport is crucial to suppress photomorphogenesis in the dark and to restrict the activity of phyA to far-red light. Another essential step in the phytochromes nuclear signaling pathway is the inhibition of the CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1)/SUPPRESSOR OF phyA-105 (SPA) complex. It is required to ensure the photomorphogenic development in presence of light. COP1/SPA represses light signaling in the dark and is inactivated by light-activated phytochromes. Presented in the current work is a phyA/phyB-dependent mechanism to inhibit the COP1/SPA complex. This relies on phyA specific interaction with SPA1 and other SPA proteins, which results in reorganization of the COP1/SPA complex. Moreover, recent findings regarding alternative mechanisms that contribute to restrain COP1 activity are discussed. Additional aspects reviewed here are the role of SPA in light-regulated processes, how SPAs link light-activation of photoreceptors and downstream signaling as well as the evolutionary origin of SPAs.

ZUSAMMENFASSUNG

Die rot/dunkelrot Photorezeptoren der Phytochromfamilie sind wichtig für die Regulierung einer Vielzahl physiologischer und entwicklungsrelevanter Prozesse in Pflanzen, so z.B. bei Keimung, Wachstum und Anpasssung an die Umwelt. Innerhalb der in *Arabidopsis thaliana* vorkommenden fünf Phytochrome spielen Phytochrom A und B (phyA and phyB) eine dominante Rolle. Die lichtvermittelte Translokation von phyA in den Zellkern benötigt die beiden funktionell homologen Proteine FAR-RED ELONGATED HYPOCOTYL1 (FHY1) und FHY1-LIKE (FHL) und ist eine der Schlüsselkomponenten der Dunkelrot-Signaltransduktion. Neben dieser Funktion als phyA-Transportproteine, wurde FHY1 und FHL eine Rolle bei der Assemblierung von phyA-Signalkomplexen im Kern zugesprochen. Die hier vorgestellte Untersuchung von Arabidopsis Mutanten gibt Einblicke in die Rolle von FHY1/FHL in der phyA-abhängigen Signaltransduktion im Kern. Die wichtigsten Schlussfolgerungen sind: (i) FHY1/FHL werden für die Dunkelrot-Signaltransduktion im Zellkern nicht benötigt; (ii) der FHY1/FHL-vermittelte Kerntransport von phyA trägt dazu bei, die Photomorphogenese im Dunkeln zu unterdrücken und die phyA Aktivität auf den dunkelroten Bereich des Lichtspektrums zu beschränken. Ein weiterer Schritt in der Phytochrom-Signaltransduktion ist die Inhibierung des CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1)/SUPPRESSOR OF phyA-105 (SPA) Komplexes, die für die photomorphogenetische Entwicklung im Licht essentiell ist. COP1/SPA unterdrückt die Signaltransduktion im Dunkeln; im Licht wird der Komplex durch Phytochrome inaktiviert. Die vorliegende Arbeit zeigt, dass diese Inaktivierung auf der spezifischen Interaktion von phyA mit SPA1 sowie weiteren SPA Proteinen basiert. Dieser Prozess führt zu einer Umformung des COP1/SPA Komplexes. In dieser Arbeit werden darüber hinaus kürzlich publizierte Ergebnisse zu alternativen Regulationsmechanismen der COP1 Aktivität vorgestellt. Weitere Aspekte, wie z.B. die Rolle von SPA Proteinen in lichtabhängigen Prozessen, die SPA-abhängige Verknüpfung von Photorezeptor-Aktivierung und Signalleitung, sowie die Evolution der SPA Proteine werden im Detail diskutiert.

1 INTRODUCTION

Due to their sessile nature, plants have developed elaborate sensory and signaling systems that enable them to adapt rapidly to ever changing environmental conditions. Within all exogenous factors sensed by plants that results in adjustment in growth and development, light is certainly the most important one. Not only is light an energy source for photosynthesis, it is also used as an important informational cue to control a wide range of physiological processes. All light-regulated responses such as seed germination and development, leaf expansion, stem elongation, phototropism, stomata and chloroplast movement, shade avoidance, circadian rhythms, and flowering time which affect plant development are conventionally included in the general term photomorphogenesis (Kami et al., 2010; Li et al., 2011). Detecting different aspects of their light environment, such as light intensity, quality, direction, and temporal patterns, plants can collect circadian, seasonal and positional information (Kami et al., 2010; Leivar and Quail, 2011; Li et al., 2011).

In order to sense light plants possess several classes of light-responsive proteins called photoreceptors. These typically contain a prosthetic cofactor or chromophore that allows them to perceive and respond to specific wavelengths of light. So far, five classes of photoreceptors, collectively detecting wavelengths from ultra-violet B (UV-B) to the near infrared, have been identified in higher plants.

The UV RESISTANCE LOCUS 8 (UVR8) monitors UV-B wavelengths (280-315 nm) (Kliebenstein et al., 2002; Rizzini et al., 2011), whereas responses to blue (B)/ultraviolet-A (UV-A) region of the spectrum (320-500 nm) are mediated by the three LOV/F-BOX/KELCH-REPEAT proteins ZEITLUPE (ZTL), FLAVIN-BINDING KELCH REPEAT F-BOX (FKF), and LOV KELCH REPEAT PROTEIN 2 (LKP2) (Christie et al., 2014; Galvao and Fankhauser, 2015). Furthermore, a number of green light responses in plants might be mediated by a yet-to-be-identified photoreceptor (Zhang and Folta, 2012). Two well characterized classes of photoreceptors, the cryptochromes (CRYs) and the phototropins (PHOTs), mediate plant responses to blue light (B) (390–500 nm) (Christie et al., 2014; Galvao and Fankhauser, 2015) while, within the whole visible light

spectrum, longer wavelengths are sensed by phytochromes (phys), which have absorption maxima in red (R) and far-red (FR) light (600-750 nm). In 1959, after the first observation of a R/FR photoreversible effect on lettuce seed germination, in which R and FR light are promoted and inhibited respectively (Borthwick et al, 1952), Butler et al. extracted the never-before-isolated R/FR light-absorbing pigment, known as phytochormes, in plants. Since then, great progress has been made in defining its biochemical properties and the molecular events that trigger phyochrome responses.

1.1 PHYTOCHROMES FUNCTION AS RED/FAR-RED PHOTORECEPTORS

In the model organism *Arabidopsis thaliana*, there are five phytochromes, designated phytochrome A to phytochrome E (phyA-E) (Xu et al., 2015). These are encoded by a small gene family and can be divided into two groups according to their protein stability in light. These groups are frequently referred as type I, or “light-labile” phytochrome, which are predominantly in dark-grown etiolated seedlings, and type II or “light-stable” phytochrome, mainly present in light-grown tissue. Whereas phyA is the only type I member, phyB-E are all type II phytochromes (Sharrock and Quail, 1989; Sharrock and Clack, 2002). Among the members of the phytochome family, phyA and phyB are the most prominent and possess different functions.

At the molecular level, plant phytochromes occur as homodimeric complexes. Each monomer consists of an N-terminal photo-sensing module (PSM) and a C-terminal output module (OPM) connected by a flexible hinge region. The N-terminal PSM, which is responsible for the absorption of light, comprises a PAS (Period/Arnt/Single-Minded) domain, a GAF (cGMP phosphodiesterase/adenylyl cyclase/FhlA) domain and a PHY (Phy-specific) domain (Figure 1.1) (Burgie and Vierstra, 2014). Employing the lyase activity intrinsic to the GAF domain (Wu and Lagarias, 2000) the plant phytochrome apoproteins covalently binds the linear tetrapyrrole chromophore, called phytochromobilin (PΦB), through thioether linkage to a conserved cysteine (Cys 357 in At-phyB and Cys 323 in At-phyA) (Burgie and Vierstra, 2014). Interestingly, this PAS/GAF/PHY tridomain photosensory core is conserved in different organisms, including many algae, fungi and bacteria suggesting its functional importance (Rensing et al., 2016). The OPM domain can be divided into two subdomains: the PAS-related

domain (PRD) containing two PAS repeats that might be used as a platform for the interaction with downstream effectors and the histidine kinase-related domain (HKRD), which does not possess kinase activity (Figure 1.1). Unlike bacterial phytochromes, in plants these C-terminal domains are not essential for signaling but serve only to enhance dimerization and possibly nuclear import (Burgie and Vierstra, 2014).

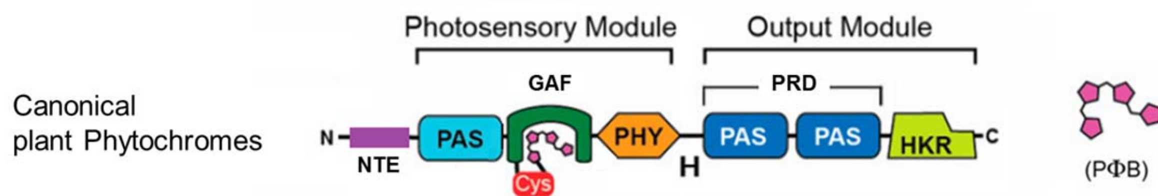

FIGURE 1.1: DOMAIN STRUCTURE OF CANONICAL PLANT PHYTOCHROMES.
N-terminal extension (NTE), N-terminal photosensory Module (PSM) consisting of Per-Arnt-Sim (PAS), cGMP phosphodiesterase/adenyl cyclase/FhlA (GAF) which binds the chromophore called phytochromobilin (PΦB) to a conserved cysteine and phytochrome-associated (PHY). This N-terminal domain is connected by a flexible hinge region (H) to the C-terminal output module (OPM). The latter contains the PAS-related domain (PRD) containing two PAS repeats and the histidine kinase-related domain (HKR). (Adapted from Burgie and Viestra, 2014; reprinted with permission, ©Copyright 2014 by American Society of Plant Biologists)

After their cytosolic synthesis, the phytochrome apoproteins can autocatalytically bind the chromophore generating their holoproteins in the inactive Pr state (Li et al., 2011). A distinctive feature of phytochrome molecules is that they can exist in two spectrally different, interconvertible forms: the inactive Pr form with maximal absorption in R light (~660 nm), and the biologically active Pfr state, which has its absorption peak in FR light (~730 nm) (Mancinelli, 1994) (Figure 1.2). Because light triggers the photoreversible conversion between Pr and Pfr conformers, which depends on the chromophore, phytochomes can work as an R/FR-dependent molecular switch (Sharrock, 2008).

It has been suggested that a possible mechanism for phytochome photoconversion involves a *Z*- to- *E* isomerization around the C15–C16 double bond between the C and D pyrrole rings of the chromophore resulting in the FR-absorbing Pfr form (Nagatani, 2010). The transition of Pr to Pfr, after light absorption, results in a change of the chromophore form followed by conformational rearrangements of the protein backbone which lead to the generation of the active Pfr form of the phytochrome (Ulijasz et al.,

2010). In a similar way, the active Pfr state can back-revert to the inactive Pr after absorption of light. Moreover, because the absorption spectra of the two forms partially overlap, the phytochrome pool is never fully converted to the Pfr or Pr following R or FR irradiation. Thus, irradiation with light results in a dynamic, wavelength-specific equilibrium between the active and inactive form of phytochromes (Figure 1.2).

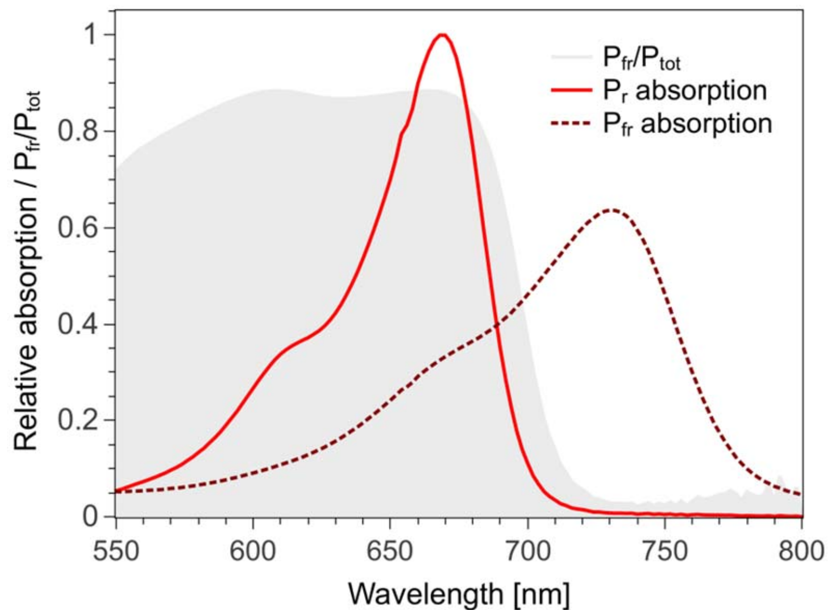

FIGURE 1.2: ABSORPTION SPECTRA OF THE INACTIVE (PR) AND ACTIVE (PFR) FORMS OF PHYTOCHROME AND WAVELENGTH-DEPENDENT PROPORTION OF PHYTOCHROME IN THE PFR FORM.

Plotted are the peaks of relative absorbance occuring for Pr (at 666nm) in R and for Pfr (at 730nm) in FR. The two Pr and Pfr conformers have partially overlapping absorption spectra. The gray area represents the proportion of phytochrome in the Pfr form at photoequilibrium in relation to the wavelength (calculated after Mancinelli, 1994).

Due to absorption of photosynthetic pigments in the R but not FR range of the visible light spectrum, the relative proportions of R and FR light varies dramatically depending on the position of the plant within the community (i.e. canopy shade versus open habitat). Using phyB plants can translate a R:FR ratio into a specific Pfr/Ptot (Ptot= Pfr +Pr) level. Low Pfr/Ptot as present in canopy shade result in de-repression of shade avoidance response, which includes for instance increased stem and petiole growth and acceleration of flowering (Halliday et al., 2009).

1.2 MODES OF ACTION OF PHYTOCHROMES

Phytochromes have been shown to regulate a multitude of physiological reactions in plants. These phytochrome responses can be distinguished into various types depending on the amount and duration of light required to induce them.

Three different action modes are known for phytochromes which are based on their sensitivities to fluence: very-low-fluence responses (VLFRs), low-fluence responses (LFRs), and high-irradiance responses (HIRs) (Li et al., 2011; Casal et al., 2014). The VLFR responses are induced by very low amounts of light (fluencies form 0.001 µmol m^{-2} to 0.05 µmol m^{-2}) of any wavelength. The minute amount of light needed to induce VLFRs establish a very low Pfr/Ptot ratio converting less than 0,02% of the total phytochrome to Pfr. Differently, the phytochrome responses, which are initiated starting from a fluence of 1 µmol m^{-2}, and saturate at about 1000 µmol m^{-2}, are referred to as LFRs. These which are induced by moderate to high Pfr/Ptot ratios, are established by R, and reversed when FR treatment is immediately followed. Therefore, they have been described as typically R/FR photorevertible responses (Li et al., 2011). Both VLFRs and LFRs follow the reciprocity law and can be induced by light pulses. In contrast, HIRs require continuous exposure to light of relatively high irradiance and saturate at higher fluences (>1000 µmol m^{-2}) than LFRs. Importantly, these responses do not follow the reciprocity law and are proportional to the irradiance until the responses are saturated. HIRs strongly depend on the light quality; in particular those wavelengths that maintain a low phytochrome Pfr/Ptot ratio (i.e. FR light) efficiently induce HIRs (Casal, 1998; Li et al., 2011).

Various types of phytochrome responses are mediated by different phytochromes. Specifically, LFRs are mediated by type II phytochromes, in particular phyB, (Casal et al., 2003; Rausenberger et al., 2011) whereas VLFR and HIR are mediated by type I phytochromes, namely phyA (Casal, 1998). These photobiological action modes partially reflect the contribution of different members of the phytochrome family to different physiological responses (discussed in section 1.5). It has come to light that the control of developmental processes requires, at molecular level, a complex interplay of plant signal transduction components. Moreover, different experimental evidences indicate that phytochrome controlled photomorphogenesis not only requires the combination and

coordination of molecular, cellular and biochemical processes, but they also take place in different subcellular compartments (Lorrain et al., 2006; Casal et al., 2014; Wang and Wang, 2015; Xu et al., 2015).

1.3 PHYTOCHROMES LIGHT-REGULATED SUBCELLULAR LOCALIZATION

The current idea is that phytochromes must enter the nucleus to initiate most light responses pointing out the essential role of this event (Fankhauser and Chen, 2008; Li et al., 2011). Prerequisite for the translocation into the nucleus is the photoconversion of phytochromes from inactive Pr, localized in the cytosol, to the active Pfr form.
Interestingly, phyA and phyB differ significantly in their nuclear import mechanisms and nuclear accumulation kinetics (Klose et al., 2014). In contrast to the very rapid (within minutes) nuclear translocation of phyA, the import of phyB occurs much slower (within hours). PhyB nuclear translocation is R/FR light-reversible, a typical characteristic of LFR, and efficiently induced by continuous R light (Li et al., 2011). The specific mechanism for the nuclear translocation of phyB depends on the presence of a nuclear localization signal (NLS)-like motif, rather than a typical NLS, localized in the C-terminal domain (Sakamoto and Nagatani, 1996; Matsushita et al., 2003; Chen et al., 2005; Fankhauser and Chen, 2008; Klose et al., 2014). The N-terminal photosensory domain of phyB interacts strongly with the C-terminal NLS domain when phyB is in the Pr conformer. Light-mediated phyB activation results in conformational changes of the photoreceptor which restores the accessibility of the NLS for the interaction with the importin-based nuclear import machinery (Chen et al., 2005).
However, this model, which is based on the presence of an intrinsic NLS-like motif is not consistent with the slow kinetic of phyB nuclear accumulation. Therefore, it has recently been proposed that the Pfr form of phyB might bind an NLS-bearing protein that co-transports phyB into the nucleus. Indeed, recently it has been shown that the PHYTOCHROME INTERACTING FACTOR 3 (PIF3), a basic helix–loop–helix (bHLH) transcription factor, can transport phyB into the nucleus (Pfeiffer et al., 2012). However, despite the presence of this PIF-dependent nuclear import mechanism it cannot be excluded that also other NLS-bearing proteins interacting with phyB might contribute to phyB nuclear transport.

It is possible, that these two different mechanisms for phyB nuclear import are not mutually exclusive. The contribution of different nuclear transport facilitators to shuttling of phyB may depend on different light quality and quantity, or the cytosolic photoreceptor-transcription factor interaction might accelerate the import.

In contrast phyA, which cannot be detected in nuclei of etiolated seedlings, is rapidly translocated in the nucleus in response to all light qualities (R, B and FR), with continuous FR light being the most effective. The ability to translocate into the nucleus under light conditions establishing low or very low Pfr/Ptot ratios, which trigger FR-HIRs and VLFRs, is an exclusive feature of phyA owing a specific phyA nuclear import system (Fankhauser and Chen, 2008; Kami et al., 2010). PhyA nuclear accumulation is strictly dependent on two plant specific proteins called FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and FHY1-LIKE (FHL) with FHY1 playing the predominant function (Hiltbrunner et al., 2005; Hiltbrunner et al., 2006; Rosler et al., 2007). These two small related proteins (202 and 181 amino acids, respectively) contain widely conserved parts: a functional NLS, a putative NES (nuclear export signal) in their N-terminal region and a phyA binding-site at the C-terminus. The latter consists of a small motif, of only 36 amino acids, which is both necessary and sufficient for FHY1 and FHL to bind to phyA (Hiltbrunner et al., 2006; Rosler et al., 2007; Genoud et al., 2008; Pfeiffer et al., 2012; Klose et al., 2014). According to the currently accepted model, the Pfr conformer of phyA binds to its transport facilitators in the cytosol and, as a complex, enters the nucleus via the general nuclear import machinery. In this compartment light is required in order to reconvert Pfr into the inactive Pr and subsequently to release FHY1/FHL from the complex. Because the phyA amount considerably exceeds the amount of FHY1 and FHL in dark-grown seedlings, the continuous photoconversion cycles are not only required to recycle back a certain level of FHY1/FHL into the cytosol in order to maintain the transport of phyA into the nucleus, but also for accumulation of a pool of active phyA in the nucleus (Rausenberger et al., 2011).

1.4 THE PHYTOCHROMES SIGNALING PATHWAY

In general, phytochromes nuclear translocation is essential to initiate a signaling cascade. This ultimately results in a specific alteration in the gene expression leading to

adjustment of growth and development. Although in the recent years extensive progress has been done in the identification of light-regulated transcriptional network organization, many questions regarding different molecular events remain open.

A significant portion of the genome, roughly 20% in *Arabidopsis thaliana*, display differential expression depending on whether seedlings are growing in darkness (skotomorphogenesis) or in the presence of light (photomorphogenesis) (Jiao et al., 2007). Seedlings that followed the skotomorphogenic development display closed cotyledons, an apical hook and elongated hypocotyl. This developmental program permits young seedlings to grow rapidly in darkness, in order to reach the sunlight. Skotomophogenic growth depends on energy stored in the seeds and therefore can be sustained for only a limited period of time. On the contrary, during photomorphogenic development, light is required on the one hand to inhibit hypocotyl elongation, and on the other hand promote both cotyledon expansion and chloroplasts development. All this permits the seedling to adapt for optimal light-harvesting capacity and autotrophic growth. During the transition from dark to light, also called de-etiolation process, plants need to actively restrain the action of repressors, which allow the skotomorphogenic development in absence of light, in order to induce the photomorphogenesis program (Xu et al., 2015). Mechanistically phytochromes tightly regulate this process by binding and inhibiting these repressors.

The first class of negative regulators of photomorphogenesis is represented by the transcription factors PIFs. All PIFs (seven in Arabidopsis) bind to the biological active Pfr form of the phytochromes with differential affinities. This interaction is mediated by the presence of a conserved active phytochrome B-binding (APB) domain located at the N terminus of PIFs. Two PIF members, PIF1 and PIF3, also interact with photactivated phyA through a less conserved APA (active phytochrome A binding) motif (Leivar and Monte, 2014). In light, the direct physical interaction of PIFs with the photoactivated, nuclear localized Pfr conformer of phytochromes leads to phosphorylation, rapid ubiquitination and proteasome-mediated degradation of PIFs which, in turn, causes a reduction of their abundance and consequently affects their ability to bind to target genes (Figure 1.3). This represents the mechanism by which phytochromes release the repression of photomorphogenesis imposed by PIFs promoting light responses. On the

other hand, in dark conditions, PIF proteins accumulate in the nucleus and promote the etiolated development regulating the expression of specific genes (Figure 1.3).

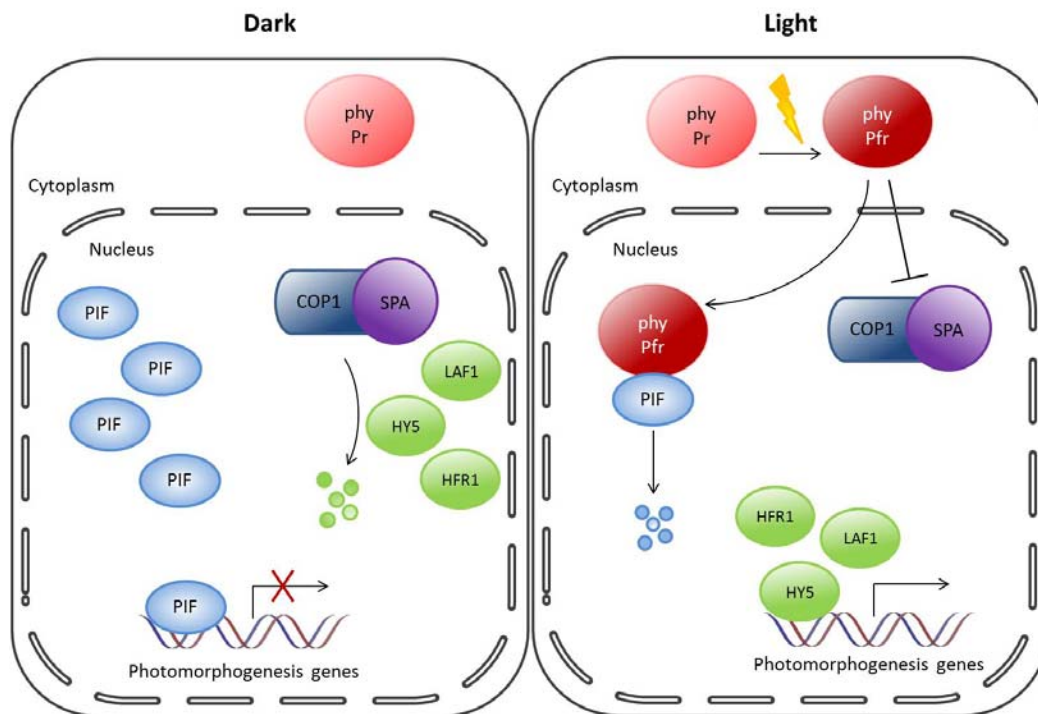

FIGURE 1.3: SIMPLIFIED MODEL OF THE PHYA LIGHT SIGNALING PATHWAYS.
In the dark, phytochromes exist in the biologically inactive Pr form. SPA proteins strongly interact with the E3 ubiquitin ligase COP1, build a complex which targets the positive regulators such as HY5, HFR1 and LAF1 for degradation. On the other hand the PIF transcription factors, acting as repressors of photomorphogenic development, remain stable and induce the etiolated form of the seedlings. Under light, phytochromes photoconvert from the inactive Pr form to the active Pfr form and translocate into the nucleus. There the photoreceptors mediate the degradation of PIFs and suppress the activity of the COP1/SPA complex. As a result, the positive regulators (HY5, HFR1 and LAF1) can accumulate and promote the photomorphogenic development.

In addition to the central role of PIFs in phytochrome signaling, PIF transcription factors are also involved in different other signaling pathways (Leivar and Quail, 2011; Casal et al., 2014; Leivar and Monte, 2014; Xu et al., 2015).

The other group of key repressors of photomorphogenesis in dark is represented by the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and members of the SUPPRESSOR OF phyA-105 (SPAs) protein family. All four SPA family members present in Arabidopsis interact, as homo- or hetero-dimers, directly with COP1. This

heterogeneous core complex, consisting of two COP1 and two SPA proteins, functions as substrate receptor of the multimeric CULLIN 4 (CUL4)/DAMAGED DNA BINDING 1(DDB1)/ RING BOX 1(RBX1) E3 ligase complex (Lau and Deng, 2012). For simplicity we refer to it in the following as the COP1/SPA complex. In dark-grown seedlings this complex targets diverse positive regulators of photomorphogenesis, such as the basic leucine zipper transcription factor ELONGATED HYPOCOTYL 5 (HY5), the atypical bHLH factor LONG HYPOCOTYL IN FAR-RED 1 (HFR1) as well the Myb transcription factor LONG AFTER FAR-RED LIGHT (LAF1) for ubiquitin-mediated degradation (Figure 1.3) (Casal et al., 2014). In response to light, phyA, as well as phyB and CRYs, inhibit the COP1/SPA complex promoting the photomorphogenic response. For this inactivation plants seem to employ different mechanisms. The first has been proposed to involve the light-dependent nuclear export of COP1. In a wavelength-specific manner phytochromes or cryptochromes seem to promote COP1 nuclear export. Although the kinetic of this process seems to be faster than the originally assumed 12 hours, it is still controversial if this mechanism reflects the very rapid responses such as activation of early light response genes (von Arnim and Deng, 1994; Osterlund and Deng, 1998; Pacin et al., 2014).

A much faster mechanism for the inactivation of the COP1/SPA complex depends on the regulation of the stability of the complex mediated by phytochromes (phyA and phyB) and cryptochromes (CRY1 and CRY2) (Lian et al., 2011; Liu et al., 2011; Zuo et al., 2011; Lau and Deng, 2012; Lu et al., 2015; Sheerin et al., 2015).

In particular, after light activation, phyB and CRYs bind to specific members of the SPA family (Lian et al., 2011; Liu et al., 2011; Lu et al., 2015; Sheerin et al., 2015). The binding between SPAs and activated phyB and CRY1 provokes the disruption of the COP1-SPA interaction, which is essential for the activity of COP1 E3 ubiquitin ligase (Saijo et al., 2003; Zhu et al., 2008). The consequence of the inactivation of the complex is a reduced ability of COP1 to promote targeting for degradation of positively-acting transcription factors (e.g. HY5, LAF1, and HFR1) which therefore can accumulate (Figure 1.3). In addition, the light-dependent reduction of SPA1 and SPA2 protein levels is another fast mechanism, possibly working in parallel to regulate COP1/SPA interaction, to reduce the activity of the COP1/SPA complex in response to light.

The COP1/SPA and PIFs regulatory pathways in the phyA signaling are however not strictly separated. Recent studies indeed provided examples for an interaction. The COP1/SPA complex indirectly regulates PIFs through the control of HFR1. In particular, COP1/SPA complex has been shown to promote the degradation of the transcription factor HFR1, which acts as negative regulator of PIF1, PIF4 and PIF5 by preventing their binding to target promoters (Yang et al., 2005; Yang et al., 2005; Lorrain et al., 2009). Also, PIF1 binds to COP1 and SPAs which in turn promote rapid degradation of PIF1 in presence of light (Xu et al., 2014; Zhu et al., 2015).
The occurrence of the above mentioned regulation points during the nuclear import, the signaling cascade and the specific light induced-gene expression, allows plants to rapidly and efficiently respond to continuous changes in the surrounding environment in order to adapt growth and development accordingly.

1.5 ECOLOGICAL FUNCTION AND EVOLUTION OF PHYTOCHROMES

Throughout the life cycle of plants, different developmental processes from germination, de-etiolation, and vegetative development to floral transition are mediated by phytochromes (Figure 1.4). This highlights the important ecological function played by the phytochromes for plant growing in a natural environment. The analyses of both individual and multiple phytochrome-deficient mutants, permitted to identify the function of individual members of the family in the control of different physiological responses. Among all photoreceptors phytochromes play a predominant role in the induction of seed germination under favorable light conditions and to restrain it when the light conditions are disadvantageous such as under deep canopy (Franklin and Quail, 2010). Germination study comparing mutants deficient in phyA, phyB, and phyA/phyB, elucidated the distinct roles of phyA and phyB in this process. PhyB plays a predominant role in regulating germination in R via the LFR mode whilst brief light pulses, which may occur during soil cultivation, are sensed by phyA and induce germination through a VLFR (Botto et al., 1996; Shinomura et al., 1996). In addition, phyA has been shown to promote seed germination in continuous FR in a HIR mode (Casal et al., 2003; Franklin and Quail, 2010). As aforementioned, seedlings that germinated beneath the soil follow the skotophotomorphogenic development.

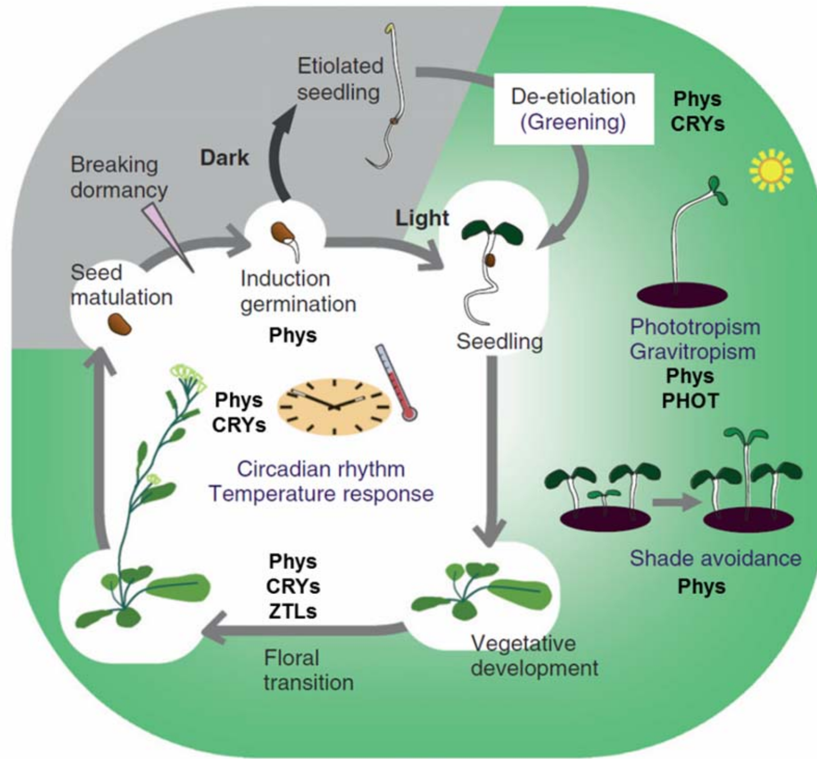

FIGURE 1.4: GROWTH AND DEVELOPMENTAL PROCESSES REGULATED BY DIFFERENT PHOTORECEPTORS.

Phytochromes display both unique and overlapping roles with other photoreceptors throughout the life cycle of plants, regulating diverse developmental processes from seed germination to floral transition. (Adapted from Kami et al., 2010; reprinted with permission, ©Copyright 2010 by Elsevier)

After emerging from the soil seedlings sense light and initiate the process of de-etiolation. This developmental stage is controlled by phytochromes, but also by cryptochromes in B light, which inhibit hypocotyl elongation, initiate the chloroplast development and promote cotyledon expansion enabling the seedling to start its photosynthetic life. This response is controlled by phyA in FR via the HIR mode whereas phyB is the predominant phytochrome regulating this process in white (W) and R light (Franklin and Quail, 2010; Kami et al., 2010).

Given that de-etiolation and vegetative development has to proceed in open habitats as well as under shade, an important function of phytochromes is to sense the presence of neighboring plants. This is particularly important because detecting a reduction of photosynthetic light caused by the presence of neighbors helps plants to change their

growth and physiology in order to reduce the degree of shade. These morphological changes include increased hypocotyl growth, stem and petiole elongation and early flowering and are conventionally called shade avoidance response (SAR) (Casal, 2012).
Shade from the vegetation is characterized by low irradiance and low R:FR ratio because R light is absorbed by photosynthetic pigments while FR is more transmitted and reflected. This R:FR ratio in the environment is translated into specific relative levels of active Pfr form (Pfr/Ptot) (Casal et al., 2014; Possart et al., 2014). The light-stable phytochromes, with phyB playing a prevalent function, suppress shade avoidance response, via LFR action mode, under high R:FR ratios (i.e. under direct sunlight). The signaling network leading to shade avoidance reactions will be discussed later in this work (in section 2.6). In contrast, the enrichment of FR wavelengths in reflected/transmitted light from surrounding vegetation can trigger enhanced phyA signaling in the HIR mode and act antagonistically to shade avoidance inducing some inhibition of elongation growth (Johnson et al., 1994; Yanovsky et al., 1995; Franklin and Quail, 2010; Casal, 2012).
Phytochromes are additionally involved in the regulation of plant architecture and gravitropic orientation. Mutants lacking in phyB display significantly elongated petioles, reduced leaf area, and increased apical dominance. In addition to this shoot morphology these plants are characterized by increased root hair growth (Nagatani et al., 1991; Reed et al., 1993). This indicates that phyB, is the primary phytochrome involved in these responses in light-grown plants. Arabidopsis hypocotyls display negative gravitropism and grow vertically against the gravitational vector in order to reach the soil surface. Mutant analyses have revealed redundant roles for both phyA and phyB in regulating light-mediated inhibition of gravitropism. Moreover, phyA deficient mutants display negative gravitropism after pulses of FR light, suggesting an important role of this photoreceptor in mediating gravitropic sensitivity in the VLFR mode (Poppe et al., 1996).
More importantly, both light and temperature influence the transition from vegetative to reproductive growth. Working in a complex regulatory network, cryptochromes, members of the Zeitlupe family and phytochromes contribute to the transition to flowering either by acting directly on key regulators of flower transition e.g. CONSTANS (CO) and FLOWERING LOCUS T (FT) or by regulating the circadian clock which

influences the photoperiodic flowering (Kami et al., 2010). Flowering time studies have shown that phyA plays a role in photoperiodic perception since the *phyA* mutants display late flowering in long photoperiods (Johnson et al., 1994; Reed et al., 1994). Pronounced late flowering was also observed in all double *phyA*, *cry1*, and *cry2* mutant combinations confirming the redundant interactions of these three photoreceptors in B-mediated floral promotion (Mockler et al., 2003). Shading (low R:FR ratio), on the contrary, leads to accelerated flowering. This specific aspect of SAR is controlled by phyB, which, under high R:FR conditions acts redundantly with phyD and phyE to repress flowering (Franklin and Quail, 2010). Interestingly, the phytochrome-mediated floral repression also depends on ambient temperature. It has been observed that by temperatures higher than 16°C *phyB* mutants display a dramatic acceleration in flowering. In this mutant the rising temperature induced an increase of *FT* expression levels. Thus, given that *FT* mRNA accumulation correlates with early flowering (Andres and Coupland, 2012), phyB can inhibit flowering through repression of *FT* expression (Halliday et al., 2003)

In conclusion, phytochromes have been shown to play a crucial role in natural light environments by monitoring the day-length which, together with temperature, provide plants important seasonal information. The day-length perception requires the integration of light signals sensed by phytochromes and cryptochromes and this is also essential to entrain the circadian clock in Arabidopsis. This, in turn, is crucial to coordinate their own reproductive development with optimal climate conditions to ensure a competitive advantage (Franklin and Quail, 2010).

Despite their functional significance, it remains an open question how phytochrome diversity across photosynthetic eukaryotes evolved. Recent studies revealed that phytochromes in some algal lineages are structurally different, including canonical and non-canonical forms, whereas in land plants, phytochrome structure is highly conserved. Moreover, the canonical plant phytochromes originated in a common ancestor of streptophytes (charophyte algae and land plants). Liverworts, hornworts and Selaginella apparently possess a single phytochrome, whereas the diversity of phytochromes in seed plants, ferns and mosses is the result of independent gene duplications that occurred within the different groups (Li et al., 2015; Rensing et al., 2016).

Phytochromes from fungi, bacteria, and algal lineages not closely related to plants (collectivily called non-plant phytochromes), exhibit altered spectral properties due to the great differences in terms of structure and employment of specific bilin as chromophore. Seed plants, ferns, nonvascular plants (liverworts and mosses) and charophyte algae possess canonical plant phytochromes and exhibit LFRs R/FR reversible action modes. This suggests that LFRs are the most basic phytochrome responses (Possart et al., 2014; Rensing et al., 2016). In contrast, only few responses resembling FR-HIRs and VLFRs (which in seed plants depend on phyA) are known in mosses and ferns. A gene duplication event during evolution of seed plants resulted in type I (phyA) and type II phytochromes (phyB and other pytochromes) and therefore, mosses and ferns do not contain phyA, though many ferns and mosses also contain multiple phytochromes (Possart and Hiltbrunner, 2013). However, different aspects regarding the molecular mechanisms of phytochromes signaling in non-seed plants are still uninvestigated. Further research on the evolutionary development of the phytochromes signaling is therefore essential to elucidate the molecular steps that regulate growth and development in lower plants.

1.6 OJECTIVE OF THIS WORK

The COP1/SPA complex is a central regulator of light-dependent plant development and in the last decades many studies were aimed at the characterization of its molecular and biochemical properties. Moreover, so far in light signaling different aspects of the tight control of this complex remain not fully understood. Thus, the purpose of my work is to contribute to the characterization of the molecular events that lead phytochromes to the inactivation of the COP1/SPA complex and thereby induction of light signaling. I focus on the identification of the molecular events, by which the interaction of light-activated phyA and phyB with different members of the SPA family, leads to the disruption of the interaction between COP1 and SPAs.

Further upstream in the phyA signaling cascade, an additional unresolved issue is the role played by FHY1 and FHL in FR signaling. Although it is well established that light-mediated nuclear translocation of phyA is one of the key steps in FR signaling and requires the function of FHY1 and FHL as nuclear transport proteins, an important aspect I aim to clarify is the nuclear function of FHY1/FHL and their role in phyA signaling.

Given that SPAs interact with cryptochromes and phytochromes and thereby repress the COP1/SPA complex, a comprehensive overview of the role of SPA proteins will be given herein. In particular, I will review the function of SPAs in light signaling and focus on different aspects including the molecular events that link photoactivated photoreceptors and downstream signaling, the contribution of SPAs in mediating wavelength specificity and the evolutionary origin of SPAs.

2 RESULTS AND DISCUSSION

2.1 REQUIREMENT OF FHY1/FHL FOR NUCLEAR TRANSPORT OF PHYA

All phytochrome-mediated changes in plants begin with the absorption of light by the photoreceptor. After photoconversion its molecular properties undergo modifications that initiate a highly regulated intracellular signaling process that ultimately results in alterations in gene transcription (Tepperman et al., 2006). In Arabidopsis, the global pattern of gene expression that emerges from previous studies using microarray-based expression profiling is that approximately 19% of all expression-detectable genes are light-regulated (Jiao et al., 2005). In particular, roughly 10% of the phytochrome-dependent light-regulated genes, described as early response genes, display a rapid change in the expression within 1 h after the onset of the light signal (Tepperman et al., 2001; Tepperman et al., 2004; Tepperman et al., 2006). However, the majority does not display alteration in expression after 3 h or more and are called late-response genes. Microarray data verified that phyA is exclusively responsible for regulation of the genes that respond to FRc signals (Tepperman et al., 2001). However, in response to R light, not only phyB, but also the remaining phytochrome family members are responsible for perception and transduction of this signal (Tepperman et al., 2001; Tepperman et al., 2004; Tepperman et al., 2006).

In dark-grown Arabidopsis seedlings phyA and phyB are predominantly located in the cytoplasm, where they are synthesized and it has been well established that light induces phytochromes localization into the nucleus. This represents a crucial step required to trigger a signaling cascade that regulates most light-responses.

As already mentioned phyA and phyB display different nuclear import mechanisms. Whereas phyB may utilize an endogenous NLS-like motif, phyA lacks an intrinsic NLS, and therefore its nuclear translocation depends on other components such as FHL/FHY1 (Hiltbrunner et al., 2005; Hiltbrunner et al., 2006; Rosler et al., 2007). Recently, it has been reported a weak induction of a light-inducible gene *PRR9*

(PSEUDO-RESPONSE REGULATOR 9) in the *fhy1 fhl* double mutants, in response to the weak R-light pulses (Pfeiffer et al., 2012). This effect has been attributed to an FHY1/FHL-independent phyA nuclear transport mediated by two members of the PIF proteins, PIF1 and PIF3. Moreover, PIF3 induced the nuclear import of phyA in a light-dependent fashion (Pfeiffer et al., 2012). Even though partially, these two TFs seems to mediate the nuclear translocation of a phyA amino-terminal fragment in a cell-free import system. However, in Arabidopsis seedlings lacking FHY1 and FHL no detectable phyA was present in the nucleus. Thus, it is currently unclear if this mechanism might operate in Arabidopsis (Klose et al., 2014). Also, this PIF-mediated phyA nuclear import can be considered a "side-effect" of the PIF-binding an phyA occurring in the cytosol. Importantly, this mechanism might contribute only in a minor part to the nuclear translocation of the photorepector however it cannot substitute FHY1/FHL-mediated phyA nuclear import.

Different studies confirmed that alteration of nuclear/cytoplasmic distribution of the phytochromes severely affected their signaling (Bae and Choi, 2008).

In phyA signaling, a validation of the essential role played by FHY1/FHL for the nuclear shuttling of phyA, comes from the phenotypic analysis of the phytochromes signaling mutants. Among these, *fhy1* is strongly impaired in phyA signaling (Whitelam et al., 1993), whereas its paralogue *fhl* exhibits a moderate phenotype (Zhou et al., 2005). The greater importance of FHY1, compared to FHL, is possibly due to its 15 times higher expression levels (Zhou et al., 2005). However, in an *fhy1* mutant, the lack of FHY1 can be compensated by expressing 3 times more FHL transcripts compared to the wild-type (Zhou et al., 2005; Yang et al., 2009).

Furthermore, as reported previously by other works, the *fhy1 fhl* double mutant behaves similar to a *phyA* null mutant in terms of two typical FR-HIRs: the inhibition of hypocotyl length, and anthocyanin accumulation. This points out that FHY1 and FHL are acting early in signaling, which is consistent with their interaction with phyA (Hiltbrunner et al., 2006; Rosler et al., 2007, Menon et al., unpublished). These above points do not discuss that phyA nuclear translocation is essential for phyA signaling and the dependence of both FHY1 and FHL for phyA nuclar import.

As previously reported by Genoud et al. (2008), only the N-terminal NLS, which is critical for the interaction with the nuclear import machinery, and the C-terminal located phyA-

interaction domain are necessary and sufficient for FHY1 functionality. However, in this study, the expression of an artificial FHY1 containing these two domains linked by an YFP-tag used as spacer has been shown to complement only the *fhy1* mutant background (Genoud et al., 2008). Thus, in order to exclude the overlapping function of FHL, we analyzed the expression of an artificial FHY1, as described in Genoud at al. (2008), in an *fhy1 fhl* double mutant. Our results demonstrated that this fusion protein is both functional *in vivo* and complements the severe *fhy1 fhl* double mutant phenotype (Menon et al., unpublished).

This indicates that FHY1 and FHL merely function as shuttle proteins and have no other signalling functions. This is supported by the work of Genoud et al. (2008). The authors showed that the expression of a constitutively nuclear localized phyA can fully restore a wild-type FR-HIR phenotype in the *phyA* and *fhy1* mutants. This is noteworthy, as in Genoud et al. (2008) the expression of a phyA-NLS has been only tested in a single *fhy1* mutant background, and therefore FHL was still present, it could not be completely excluded that FHY1 and FHL might possess an additional function further downstream in the FR signaling pathway (Yang et al., 2009; Chen et al., 2014). Therefore, we analyzed seedlings lacking functional FHY1 and FHL expressing phyA fused to a NLS. These lines clearly displayed a light- and FHY1/FHL independent nuclear localization of phyA. Moreover, the expression of a constitutively nuclear localized phyA can fully restore a wild-type FR-HIRs phenotype in the *phyA fhy1 fhl* triple mutant. This confirms that, in the FR signaling pathway, FHL and FHY1 function as essential system required for phyA nuclear transport and that this system can be replaced by simply attaching a NLS to phyA. Moreover, our results point out that FHL and FHY1 are not required for phyA downstream signaling in the nucleus (Menon et al., unpublished).

The wavelength specificity of phyA and phyB correlates perfectly with the light quality required for their nuclear import. In fact phyB is only efficiently transported into the nucleus in response to R light and inhibited by FR light and is thus a typical LFR. In contrast, very low levels of light (VLFR) and light qualities resulting in only low levels of Pfr (FR) triggers nuclear translocation of phyA (Fankhauser and Chen, 2008). Because accumulation of phyA in the nucleus is efficient under continuous irradiation with high fluence rate of FR, phyA nuclear transport itself can be considered an HIR (Possart et al., 2014). However, under this condition the relative abundance of the active Pfr is

much lower than in R light. The particular characteristic of possessing identical absorption but different action spectrum largely depends on the different mechanisms to control phyA and phyB nuclear translocation (Rausenberger et al., 2011). In the mathematical modelling presented by Rausenberg et al. (2011) several HIR modules have been identified. These ensure the sharp separation of the phyA action peak from phyB. Based on theoretical analysis, the specific phyA action peak in FR light requires on the one hand that the Pfr conformer is degraded at a rate significantly higher than the Pr, on the other hand the Pfr-dependent enhanced stability of the FHY1/FHL transporter-phyA complex. At the same time, the transition back to the Pr state represents an indispensable requirement for the efficient dissociation of the complex and the nuclear accumulation of the photoreceptor (Rausenberger et al., 2011). One of these HIR modules that contribute to maintain the shift of maximal action from R to FR is represented by the cytosolic and nuclear Pr/Pfr photoconversion cycles, which operate in opposite directions (Rausenberger et al., 2011) (Figure 2.1).

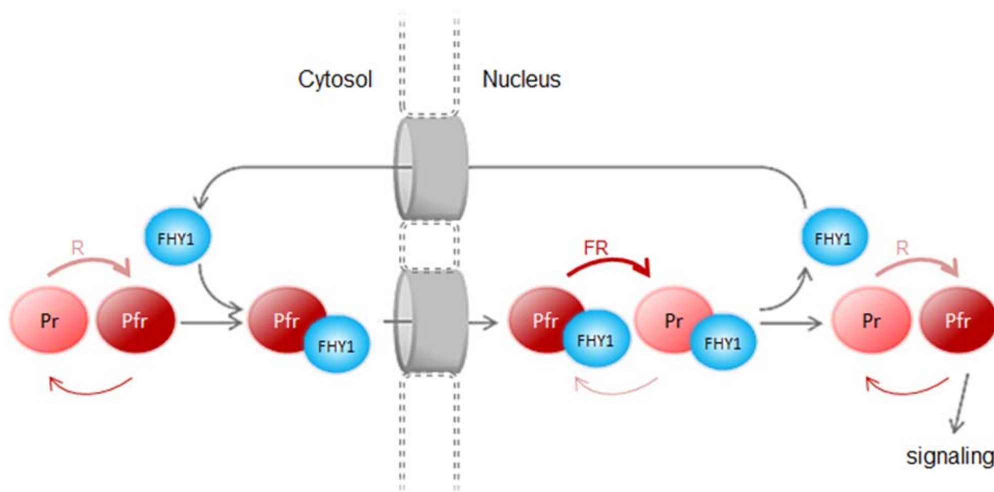

FIGURE 2.1: PHYA NUCLEAR TRANSLOCATION DEPENDS ON TWO FUNCTIONAL HOMOLOGS FHY1 AND FHL.

Upon light exposure, the Pfr form of phyA is imported into the nucleus by the binding with FHY1/FHL. For proper FR perception a constant photocycling between the active (Pfr) and inactive state (Pr) is essential. Two antagonistic photoconversion cycles, one in the cytosol and one in the nucleus are occurring. The latter furthermore ensures FHY1 cytosolic recycling. (Adapted from Possart et al., 2014; reprinted with permission, ©Copyright 2014 by Elsevier)

Fusing an NLS directly to phyA bypasses the FHY1 import cycle and results in hypersensitivity to FR and R light, although in R light the effect is stronger. Our data suggest that the phyA action peak in the phyA-NLS expression lines is much extended into the R light range than in the wild-type, which is in line with the prediction by Rausenberg et al. (2011) (Menon et al., unpublished).

Our results suggest that the control of phyA nuclear accumulation plays an important role in restricting phyA activity to the FR range of the light spectrum. Thus, the unique ability of phyA to respond more strongly to FR light is not a property of the phyA photoreceptor itself, but a property of the phyA signaling network.

Remarkably, different to wild-type plants, phyA-NLS expressing plants display a *cop* phenotype. This indicates that FHY1/FHL transport system is important to prevent high levels of phyA present in the nucleus under unfavorable conditions (i.e. in darkness).

In order to maintain the maximal response in FR light, in addition to the requirement of rapid phyA degradation in R light, it has been proposed that a phyA-mediated phosphorylation of FHY1, but not FHL, in R light is required (Shen et al., 2009). Under this light condition FHY1 becomes gradually phosphorylated by action of the Pfr form of phyA (Shen et al., 2009). This phosphorylated FHY1 binds to phyA but is retained in the cytosol thereby inhibiting further phyA nuclear accumulation and consequently phyA nuclear signaling (Chen et al., 2012).

An additional possible molecular mechanism for shifting the phyA action peak from R to FR might depend also on the dimerization properties of the photoreceptor.

In line with this hypothesis, a recent study has described a mechanism how the phyB action is abolished in FR. This depends first on the formation of subnuclear foci, called nuclear bodies (NBs), which represent functionally relevant structures for the phyB signaling (Klose et al., 2015). Given that the formation of NBs is strictly Pfr dependent, it has been shown that Pfr–Pfr homodimers dissociate very slowly from the NBs, whereas the dissociation rate of Pfr–Pr is fast. Thus, the NB association and dissociation rates of the Pfr–Pr heterodimers have been identified as critical parameters for this mechanism (Klose et al., 2015). Experimental evidence has indicated that NBs serve as nuclear Pfr storage sites stabilizing the Pfr form of phyB. Suppression of dark reversion in the NBs could explain the long persistence of Pfr containing NBs. Thus, the rapid dark reversion of unbound heterodimer Pfr–Pr furthermore diminishes the probability that this dimer

species can induce signal transduction suggesting that only the Pfr–Pfr conformer seems to be able to initiate signal transduction (Klose et al., 2015). In the case of phyA given that Pfr–Pfr homodimers are only present in R light, whereas the relative amount of Pfr–Pr heterodimers is highest in FR light, it seems possible that plants suppress the activity of phyA in R by preventing nuclear transport of Pfr–Pfr homodimers. This would implicate that, in the case of phyA, Pfr–Pr heterodimers are physiologically active and efficiently transported in the nucleus; in contrast Pfr–Pfr homodimers might be retained in the cytosol. Here the Pfr–Pfr homodimers forms sequestered areas of phytochrome (SAPs), in R light, which have been thought to represent phyA-specific cytoplasmic foci involved in degradation/storage of light- activated phyA. Thus, the antagonism between the nuclear transport, in FR, and recruitment in the SAPs, occurring in R, could determine the amount of phyA present in the nucleus. Which of these two processes prevails, nuclear translocation or localization in the SAPs is in turn wavelength-dependent.

2.2 FUNCTION OF FHY1/FHL IN PHYA NUCLEAR DOWNSTREAM SIGNALING

Beyond its function in translocating phyA into the nucleus, FHY1 has been indicated to guide phyA to associate with the promoter regions of FR-responsive genes (such as chalcone synthase, *CHS*) and bridge the association between phyA and transcription factors (e.g. LAF1 and HFR1) for target gene transcription (Yang et al., 2009).
The idea of a nuclear function of FHY1 in gene expression has also been supported by the fact that FHY1 together with phyA, is recruited to the DNA via the transcription factors (TFs) HY5 and PIF3 and form a complex to co-activate the transcription (Yang et al., 2009; Chen et al., 2014). More recently, ChIP-seq analysis confirmed the phyA and FHY1 co-action on the promoters of a wide spectrum of target genes (Chen et al., 2014). Nevertheless, since the authors of this report identified genes that are uniquely associated with phyA or FHY1, they concluded that phyA and FHY1 can also perform independent functions in response to enriched FR light signals (Chen et al., 2014).

Both phyA and FHY1 lack a DNA binding domain, therefore, they depend on TFs for the association with *cis*-elements and for transcriptional regulatory activities. Thus it is possible that the specificity of *cis*-elements and the affinity of their corresponding TFs for phyA and FHY1 interaction could be crucial for the different coordination or unique phyA/FHY1 working patterns on associated gene promoters. Despite that it should be pointed out that Chen at al. (2014) did not explain the possible mechanism of a FR-induced phyA-indipendent FHY1-DNA association. Also, because the phyA recruitment to the DNA was analyzed only in an *fhy1* single mutant, it could not be excluded that this depends on FHL. The long hypocotyl phenotype in FR of the *fhy1 fhl* double mutant can be rescued by the expression of a constitutively nuclear localized phyA, as shown in our report. This indicates the presence of a FHY1/FHL-independent phyA nuclear signaling pathway. Moreover, an artificial FHY1 (NLS-YFP-FHY1$_{166\text{-}202}$) can restore the wild-type phenotype of the *fhy1 fhl* double mutant, pointing out that FHY1/FHL function essentially as shuttle proteins (Menon et al., unpublished). In addition to that, phenotypic analyses, performed in our work, show that phyA-NLS expressing seedlings still require phyA downstream signaling components, such as HY5 and HFR1 for a full response to FR light (Menon et al., unpublished). Theoretically, phyA downstream signaling components, such as the transcription factor (TF) PIF3, may compete with FHY1/FHL for the same binding site to phyA within the nucleus. Alternatively, FHY1/FHL and TFs may bind simultaneously to different motifs of phyA generating a pool of transient multimeric protein complexes. As yet another alternative, different binding affinities of TFs and FHY1/FHL, could also affect each others binding, inducing conformational changes of this complex which trigger the FHY1/FHL dissociation. However, existence of any, or all, of these molecular events needs to be determined by further investigations.

The important conclusion of our findings is that FHY1 and FHL are not essential for phyA downstream signaling in the nucleus and that might at best play a modulation role in phyA signaling in the nucleus.

2.3 POSSIBLE CYTOSOLIC FUNCTION OF PHYA

From the microarray data discussed above it emerges that phytochrome-mediated changes in gene expression generally require several minutes, if not hours, to take effect (Tepperman et al., 2001; Tepperman et al., 2006). However, different examples have been reported in which phytochrome-mediated responses occur within few minutes or even seconds and therefore are considered far too fast to involve the translocation in the nucleus and changes in the transcriptome. In addition, because phytochrome-translocation into the nucleus is rather slow, except for phyA, the majority of the intracellular active Pfr pool is not nuclear localized (Nagy and Schafer, 2002).

For these reasons it has been suggested that phytochromes may activate signaling pathways in both nucleus and the cytoplasm.

Extensive kinetic studies performed in the last decades revealed the presence of phytochrome-dependent signaling routes in the cytoplasm. An example is provided by the sleep movements of leaves in different legumes, such as sleepy plant (*Mimosa pudica)*, persian silk tree (*Albizia julibrissin*) and rain tree (*Albizia saman*) (Hughes, 2013). The change in leaflet angle is triggered by massive osmotic changes in the specialized cells of the pulvinus (a characteristic structure at the base of the petiole). Moreover, in these plants R light induces closed leaflets to rapidly (2-10min) open and this effect can be reversed by a FR pulse indicating the involvement of type II phytochromes in this process (Fondeville et al., 1966; Hillman and Koukkari, 1967; Koukkari and Hillman, 1968; Hughes, 2013). Thus, light-stable phytochromes, besides inducing drastic osmotic changes, can also rapidly alter the bioelectric potential of the cells in an R/FR reversible way. This results in responses that occur as rapid as 30s such as reported in experiments performed on excised root tips of barley (*Hordeum vulgare*), in mung bean (*Phaseolus aureus*) and in coleoptiles of oat (*Avena sativa*) (Jaffe, 1968; Tanada, 1968; Newman and Briggs, 1972; Hughes, 2013). The fastest phytochrome cytoplasmic response, measurable within 2-3 s, reported so far regards the R/FR reversible cytoplasmic motility observed in epidermal cells of the aquatic plant *Vallisneria gigantea* (Takagi et al., 2003).

Most of these fast responses display a characteristic R/FR photoreversibllity and occur in adult plants suggesting that rather type II phytochrome (phyB-E) than phyA might be involved.

More recently Rösler et al (2007) provided evidences of cytoplasmic responses regulated specifically by phyA. Using mutants in which the phytochrome nuclear translocation is defective such as *fhy1 fhl* double mutant seedlings, the authors analyzed the phyA:GFP migration behavior and the phenotype in FR–HIR and VLFR conditions and identified phyA-mediated inhibition of gravitropism, inhibition of hypocotyl elongation in B and R-enhanced phototropism. In higher plants directional responses mediated by the membrane localized B light photoreceptor phototropin are modulated by phytochromes. Consistent with that, it has been reported the formation of phyA-PHOT1 complexes, likely mediated by a member of the PHYTOCHROME KINASE SUBSTRATE (PKS) family PSK1, at the plasma membrane as a possible mechanism for both phyA VLFR- and PHOT1-mediated phototropism (Jaedicke et al., 2012; Hughes, 2013). Kinetic studies on wild-type, *fhy1 fhl, phyA*, and phyA-NLS-GFP seedlings could show that nuclear phyA accelerates phototropism, whereas in *fhy1 fhl*, reorientation toward blue light occurred much slower than in the wild-type. These results, which do not exclude cytosolic effects of phyA on the regulation of hypocotyl phototropism, indicate however a more prominent role of nuclear phyA in the promotion of these responses by regulating nuclear gene expression (Kami et al., 2012). Thus, it seems plausible that under the tested low B light conditions residual nuclear phyA signaling in *fhy1 fhl* contributes to light-regulated gene expression.

One possible explanation could be that cytosolic phyA initiates a signaling cascade taking advantage of the phototropin-associated machinery. However, only little is known about phototropin downstream signaling components and also how phytochrome cytoplasmstic signals are transmitted. Alternatively, this remaining B light–regulated gene expression could be due to a small amount of phyA that can still translocate into the nucleus, possibly through an alternative pathway.

Despite the presence of a phytochrome-mediated cytoplasmic signaling, from a photomorphogenic point of view, it is apparent that that the majority of the phytochrome-mediated signaling events occur in the nucleus.

2.4 EVOLUTIONARY ASPECTS OF FHY1-DEPENDENT PHYA NUCLEAR TRANSPORT

As already highlighted the FHY1-dependent nuclear translocation of phyA represents an essential step for the regulation of gene expression and for phyA signaling responses. Phylogenetic analyses show that FHY1-like proteins are present in different angiosperms suggesting the pivotal role of FHY1 for seed plants (Genoud et al., 2008). In lower plants the cytoplasmic phytochrome-pool and phytochromes associated at the plasma membrane seems to regulate a large number of responses. However, recently, FR-HIR-like responses have been identified in the moss *Physcomitrella patens* and the fern *Adiantum capillus-veneris* (Possart and Hiltbrunner, 2013). These authors showed that in the moss Physcomitrella, brief light exposure triggered rapid translocation of YFP-tagged endogenous Pp-phy1 and its homolog Pp-phy3 into the nucleus; similarly to rapid nuclear translocation of phyA in seed plants (Possart and Hiltbrunner, 2013). Also, these Physcomitrella phytochromes revealed rapid degradation of the Pfr conformer. In addition, the authors showed that Physcomitrella and other cryptogams contain FHY1-like proteins. The Pp-phy1 and Pp-phy3 interact with FHY1-like proteins and display FHY1-like dependent nuclear translocation (Possart and Hiltbrunner, 2013; Possart et al., 2014). The occurrence of FHY1-mediated nuclear transport of phytochromes in both seed plants and mosses suggests that this mechanism evolved in the last common ancestor of modern seed plants and cryptogams (Possart and Hiltbrunner, 2013). As already mentioned, in the latter was present only phytochromes mediating LFR (similar to type II phytochromes) and phyA is unique to seed plant (Possart et al., 2014). Thus, it is likely that these properties, essential for FR-HIR responses, precede the diversification of type I and type II phytochromes in seed plants. The spread of plants into multiple environments required the adaptation to increasing complexity of light conditions. Thus, plants had to evolve a mechanism to separate phytochromes activity to a specific light condition. It seems therefore possible that the ancestral phytochrome of seed pants and cryptograms utilized both FHY1-dependent and –independent mechanisms for the nuclear transport. During phytochromes evolution higher plants may

have developed phytochromes, lacking NLS which therefore depend on a FHY1-dependent mechanism for their nuclear transport (Possart and Hiltbrunner, 2013). In fact, as above mentioned, the regulation of phyA nuclear abundance, controlled by FHY1 and FHL, is particularly important to restrict phyA activity to FR light conditions. Moreover, the control of phyA nuclear accumulation mediated by FHY1/FHL is essential to prevent initiation of downstream signaling in absence of light. Because phyA is very sensitive to light and even small amount of active phyA can trigger VLFRs the control of phyA nuclear accumulation is also crucial to control VLFR.

2.5 REPRESSION OF THE COP1/SPA COMPLEX IS DIRECTLY MEDIATED BY PHYTOCHROMES

A complex nuclear signaling network which includes a considerable number of positive and negative regulators functions downstream of phytochromes in light signaling. Amongst them, COP1 and SPA, which form an E3 ubiquitin ligase complex, operate as negative regulators and are essential for the repression of light signaling in darkness. In light, the inhibition of the COP1/SPA complex triggers the accumulation of TFs including LAF1, HFR1 and HY5 which act as positive regulators that promote the photomorphogenic development (Kami et al., 2010; Li et al., 2011).

With an increasing number of known targets, it is emerging the role of COP1/SPA complex as a pivotal signal integrator not only in photomorphogenesis responses but also in the crosstalk with other signaling pathways. This highlights that tight regulation of this complex represents a crucial point in plant development.

Although it is established that phytochromes must inactivate the activity of the COP1/SPA complex in etiolated seedlings, the molecular mechanism has so far been unknown. In a yeast two-hybrid (Y2H) screen we found SPA1 as a direct interactor of photoactivated phyA identifying an important molecular step leading to the inhibition of the COP1/SPA in light signaling. This light-dependent interaction was confirmed in our report also via co-immunoprecipitation in Arabidopsis and in fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) analyses in transiently transformed *Nicotiana benthamiana* leaves (Sheerin et al., 2015). In addition

we identified, in yeast and by FRET-FLIM measurements, the specific interaction of phyA with a further member of the four SPA proteins present in Arabidobsis, namely SPA2. Interestingly, we confirmed both in yeast and *in planta* a light-dependent interaction between SPA1 and the major phytochrome present in adult plants, phyB (Sheerin et al., 2015). These results indicate that both phyA and phyB have in common a similar light-dependent interaction with SPA1.

Moreover, we and others identified in yeast and *in planta*, that light-activated phyA and phyB compete with COP1 for binding to SPA1 indicating the molecular event leading to the inhibition of the direct interaction of COP1 and SPAs and consequently rapid accumulation of TFs (like HFR1) which promote photomorphogenic responses in FR and R light (Lu et al., 2015; Sheerin et al., 2015). Lu et al. (2015) confirmed that in presence of phyB, the COP1–SPA1 interaction was reduced progressively along prolonged exposure to the R light with a reduction in the amount of co-immunoprecipitated COP1 with Myc-SPA1 when compared with that in the dark. This would support the idea that phyB–SPA1 interaction facilitates the dissociation of SPA1 from COP1 (Lu et al., 2015) and phyB induces nuclear exclusion of COP1 (Osterlund and Deng, 1998). These results are consistent with our proposed mechanisms of the phytochrome-mediated inactivation of the COP1/SPA complex.

We could show that phyA also directly interacts with COP1. Thus, the binding between phyA and SPA does not result in full dissociation of the COP1/SPA complex but rather results in a reorganization of the complex by separation of the physical contact between COP1 and SPA1 (Viczian et al., 2012; Sheerin et al., 2015). Interestingly, binding of CRY2 to SPA1 strengthens the interaction of COP1 and CRY2, but nevertheless suppresses the activity of the COP1/SPA complex (Zuo et al., 2011). Differently, phyB and CRY1 promote the dissociation of the complex (Lian et al., 2011; Liu et al., 2011; Lu et al., 2015; Sheerin et al., 2015). Even if also phyB and COP1 interact with each other (Yang et al., 2001; Jang et al., 2010) it could not be excluded that phyA and phyB use different mechanisms concerning the dissociation of the complex.

However, considering the rapid reduction of the COP1–SPA1 interaction by photoactivated phyB (Lu et al., 2015), it seems likely that nuclear depletion of COP1, normally triggered by a long-time exposure to light (e.g. 12 hours), may contribute only partially to the inactivation of COP1 from SPA1 in plant cells.

Noteworthy, interactions between phytochromes, SPAs and COP1 take place in NBs, where these proteins co-localize in response to light.
Currently the precise function, formation dynamics, components and regulation of NBs are still largely unknown. Interestingly, localization studies revealed that a large number of light signaling components co-localize in NBs and that many light signaling mutants also exhibit defects in NBs formation. This indicates the functional relevance of NBs in light signaling (Van Buskirk et al., 2012). By immuno-fluorescence and immuno-electron microscopy analysis we confirmed that native Arabidopsis phyA translocates to NBs after irradiation with light and that the observed electron- dense structures of 100 to 200 nm in diameter correspond to phyA-containing NBs (Sheerin et al., 2015). However, not all proteins that co-localize in NBs interact with each other, showing that also proteins that do not directly interact with each other can co-localize in NBs. In accordance with that, even though all four SPA proteins co-localize in the NBs with phyA, the latter interacts specifically with only two of them (Sheerin et al., 2015).
Concerning the kinetics of NB formation, it is important to distinguish between “early” and “late” NBs. The first category includes the NBs that appear after few minutes of light exposure and disappear rapidly during further irradiation. The formation of these small early NBs has been shown to depend on PIFs (Kircher et al., 2011; Klose et al., 2014). Indeed, the light-dependent interaction of the PIF3 with phyA, which triggers the rapid decline of PIF3 abundance, occurs in this early type of NBs (Al-Sady et al., 2006). Moreover, the correlation between the formation of phyB early NBs and PIF3 degradation and that a NB-deficient mutant is also defective in the degradation of PIF1 and PIF3 indicate that phyB NBs are required for PIF degradation (Galvao et al., 2012; Van Buskirk et al., 2012; Klose et al., 2014). However, the molecular mechanism by which phytochrome NBs mediate PIF degradation is still unclear.
Differently, during extended irradiations a second late type of larger NBs are formed. These have been indicated to be functionally relevant structures for phytochrome signaling pathways. Notably, many positive transcriptional regulators such as HY5, LAF1 and HFR1 co-localize with COP1 in late NBs as well as members of the SPA proteins prior to their degradation (Van Buskirk et al., 2012). Thus, because the ability to form NBs often correlates with the presence of COP1 and SPAs in the NBs and degradation

of light signaling components, one widely proposed hypothesis is that NBs are involved in protein turnover of key transcriptional regulators.

In our report we showed that SPA1 localization to NBs is not dependent on light, in contrast to phyA (Sheerin et al., 2015). Thus, because two *phyA* mutants impaired in NB formation (i.e. phyA G727E and phyA E777K) are unable to interact with SPA1 we hypothesized that SPA1 could function in recruitment of phyA into NBs. However, our analysis revealed that this is not the case since phyA-translocation in the NBs remains unaffected in a *spa123* triple mutant background (Sheerin et al., 2015). Therefore, it appears likely that another factor is required for NB formation. One possible candidate could be COP1 as it is constitutively localized in the NBs and co-localizes in these compartments with many of its targets before targeting them for degradation. For this reason we investigated the phyA NB formation in a *cop1-4* mutant background. The result of our analysis was that in response to FR light phyA-translocation in nuclear speckles was also not affected in the *cop1-4* mutant. However, because the *cop1-4* mutant is a weak *cop1* mutant that expresses a truncated COP1 protein we could not completely exclude that COP1 is involved in phyA NB localization (Deng and Quail, 1992; Mcnellis et al., 1994; Sheerin et al., 2015). We confirmed, furthermore, the direct interaction of phyA and COP1 (Seo et al., 2004; Viczian et al., 2012) occurring in NBs (Sheerin et al., 2015).

Expression of both SPA1 and COP1 results in a constitutive localization of both proteins in the same NBs. This supports the idea that this type of NBs containing phyA, COP1 and SPA plays an important function in the control of protein degradation events. For this reason during dark-to-light transition this complex needs to be strictly controlled.

2.6 MULTIPLE MECHANISMS INHIBIT THE COP1/SPA COMPLEX

2.6.1 NUCLEAR/CYTOPLASMIC TRANSLOCATION OF COP1

Previously, it has been proposed that light-dependent exclusion of COP1 from the nucleus could be a possible mechanism to explain the inactivation of COP1 activity (Osterlund and Deng, 1998). This idea was also supported by the observation that

COP1 with a site-directed mutation of its single NLS (residues 293–314) enters the nucleus inefficiently, causing constitutive photomorphogenesis (Stacey et al., 1999; Stacey et al., 2000).

Differently to the former accepted idea in which both phytochromes and cryptochromes induce a slow export of a COP1-GUS fusion protein into the cytosol (Osterlund and Deng, 1998), the translocation of a smaller YFP-COP1 fusion protein out of the nucleus in response to W light occurs much faster. This suggested that this process might be important to regulate the stability of COP1 target proteins (Pacin et al., 2014).

However, our analysis of Arabidopsis seedlings (Col-0 background) expressing *pCOP1-mCherry-COP1* revealed that, under increasing FR exposure time, COP1 nuclear localization was not substantially affected (supplemental Figure S1). Moreover, we could show that the kinetic of accumulation of the target TF HFR1 in FR light does not temporally correlate with COP1 nuclear export, but rather fits to the kinetics of phyA nuclear accumulation and NB formation (Sheerin et al., 2015). Thus, it appears probable that the inhibition of HFR1 degradation depends on the phyA-induced reorganization of the COP1/SPA complex, rather than to the COP1 relocation in the cytosol.

Under deep vegetation the decrease in the ratio of R to FR wavelengths is primaryly perceived by phyB which functions in suppressing shade avoidance responses in normal light conditions. The signaling network leading to shade avoidance reactions involves, besides the phyB-PIF-auxin pathway, a second branch including the COP1/SPA complex (Pacin et al., 2013) (Figure 2.2). Whereas the major events linking PIFs to shade avoidance have been established, important aspects of phyB regulation of COP1 remain unanswered. Importantly, COP1 relocates into the nucleus in response to simulated shade light (low R:FR ratio) (Pacin et al., 2013). Under this condition, on the one hand, the reduced activity of phyB would enhance the activity of PIFs, which promote auxin synthesis, and, on the other hand, an increase in COP1 nuclear abundance would lead to a reduction of a photomophogenic response. Accordingly, we would expect a reassembly of the COP1/SPA complex in presence of shade (Figure 2.2). Therefore, we tested (in collaboration with the Group of Prof. Jorge Casal, University of Buenos Aires, Argentina, and Sven zur Oven-Krockhaus member of Prof. Harter Group, ZMBP, University of Tübingen, Germany) this idea in transiently

transformed tobacco expressing mCherry-COP1, GFP-SPA1 and phyB-LUC, via FRET-FLIM measurements under simulated sunlight vs. simulated shade (data not shown).

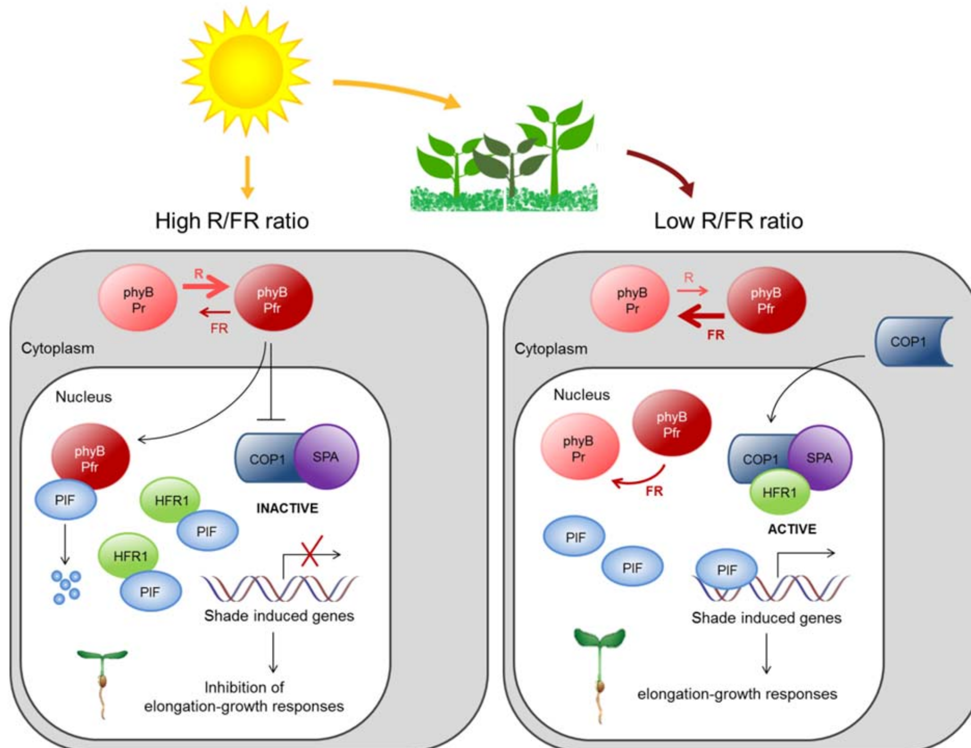

FIGURE 2.2: MOLECULAR NETWORK OF THE SHADE AVOIDANCE RESPONSE.
Left: in open habitats (high R:FR ratio), the COP1/SPA complex and the PIFs are inactivated by the active Pfr form of phyB. In addition the COP1/SPA target HFR1 can accumulate and PIFs-mediated elongation responses are inhibited. **Right:** under shade conditions (low R:FR ratios) photoequilibrium of phyB is shifted to the inactive Pr form. The nuclear COP1 abundance increases causing the reassembly of the COP1/SPA complex. The latter and the PIFs are thus activated. Also, the active COP1/SPA complex can target HFR1 for degradation. Thus, HFR1 fails to inhibit PIFs transcription activity leading to shade avoidance elongation responses (see text for references).

Our preliminary results suggest that in sunlight phyB may inhibit the COP1/SPA1 complex generated with the available nuclear COP1. Moreover, in shade the nuclear COP1 abundance increases leading to the re-assembly of the COP1/SPA1 complex as supported by a reduction of the lifetimes value compared to sunlight conditions. We could also observe a positive effect of phyB overexpression on the inhibition of the complex interaction under sunlight conditions, while the native amount of phyB in tobacco leaves was insufficient to induce this effect. Nevertheless, it remains an open question if the nuclear exclusion of COP1 is a consequence of the dissociation of the

COP1/SPA1 complex or if there exists another mechanism required to release COP1 from the complex and to export it in the cytoplasm. Interestingly, for proper COP1 localization a NLS motif is required. In addition, a NES (residues 67–177), which partially overlaps with its coiled-coil region important for the formation of homo/heterodimers with the members of the SPA proteins, is also required for proper COP1 localization (Stacey et al., 1999; Stacey and von Arnim, 1999; Stacey et al., 2000). Therefore, it could be speculated that the interaction with SPAs, which are constitutively nuclear localized (Hoecker et al., 1999; Laubinger et al., 2004) might therefore partially mask the NES and retain COP1 in the nucleus in dark. It is therefore one possibility that the binding of the light-activated phytochromes with the COP1/SPA complex induces conformational changes in the COP1 protein that expose the NES, masked by SPA in darkness, promoting the dissociation of the COP/SPA complex thereby inducing COP1 remobilization out of the nucleus. In onion epidermal cells the CONSTITUTIVELY NUCLEAR LOCALIZED SUBUNIT 1 of the COP9 signalosome, called CSN1, interacts with the coiled-coil domain of COP1 and masks its NES promoting nuclear localization of GUS-COP1 in darkness (Wang et al., 2009). Thus, the light dependent interaction with the photoreceptors could hypothetically affect the CSN1-dependent retention of COP1 in the nucleus. Alternatively, the light activated photoreceptors could trigger post-translational modifications (i.e. phosphorylation in a direct or indirect way), that leads to COP1 release from the complex and its nuclear export. However, all these possible mechanisms have never been validated experimentally. For this reason, structural studies on the COP1 protein and its interactions with photoreceptors will be important to define the roles of COP1 subcellular targeting sequences and to understand their regulation by light.

Though, we could not completely exclude that the COP1 nuclear export and phytochrome-mediated inhibition of COP1 biochemical activity both contribute to regulate its nuclear targets especially under other light conditions. It is moreover likely that the inactivation of the COP1/SPA complex mediated by phytochromes represents a fast response to light signals whereas the COP1 cytoplasmic relocation might contributes for a long-term inactivation of COP1 activity.

2.6.2 Phytochrome-mediated SPA proteins turnover

The regulation of the COP/SPA complex activity may not only depend on COP1 nuclear relocalization and cyptochrome and phytochrome-mediated reorganization of the COP1/SPA complex, which leads to inactivation of its E3 ubiquitin ligase activity. Another mechanism could also depend on the phyA- and phyB-regulated SPA1 and SPA2 protein turnover.

Four SPA proteins are present in *Arabidopsis thaliana*, which have overlapping and distinct functions regarding the regulation of photomorphogenesis. SPA1 and SPA2 are necessary to repress photomorphogenesis in the dark, while SPA3 and SPA4 only have minor contributions in darkness (Laubinger et al., 2004; Fittinghoff et al., 2006; Balcerowicz et al., 2011; Chen et al., 2015). Thus, recent studies have focused on SPA1 and SPA2 light dependent turnover (Balcerowicz et al., 2011; Chen et al., 2015). Currently it has not been reported if also SPA3 and SPA4 undergo light-mediated degradation. Notably, SPA1 and SPA2 are the only members of SPAs occurring in young seedlings and specifically interact with phyA. This could be a possible reason why SPA1 and SPA2 degradation in light is phyA-dependent.

Whereas light does not affect *COP1* transcript or COP1 protein levels (Deng and Quail, 1992; Zhu et al., 2008) the transcript levels of *SPA1*, but not of *SPA2*, are increased 1 h after exposure to R or FR (Hoecker et al., 1999; Fittinghoff et al., 2006), but both SPA1 and SPA2 are rapidly destabilized within 1 h of FR irradiation or after exposure to red and blue-light (Balcerowicz et al., 2011; Chen et al., 2015). In response to light it has been shown that SPA2 is degraded more rapidly than SPA1. As a consequence, SPA2 is no longer able to repress photomorphogenesis in the light whereas SPA1 is still functioning (Balcerowicz et al., 2011). Moreover, it has been shown that SPA2 turnover largely depends on phyA. The fact that higher SPA2 protein levels accumulate in *cop1* seedlings irradiated with FRc than in the wild-type indicates that COP1 is required for normal SPA2 turnover (Chen et al., 2015).

Because, SPA2 degradation is not remarkably affected in the *spa1 spa3 spa4* mutant background it has been concluded that either the COP1/SPA2 complex can mediate the degradation of SPA2. Alternatively, since COP1 auto-ubiquitinates *in vitro*, the

proteasomal degradation of SPA1 and SPA2 might be a result of the auto-ubiquitination activity in a SPA independent-way (Saijo et al., 2003; Seo et al., 2003).

The light-dependent interaction between phyA and COP1/SPA2 complex might result in a change of its targeted substrates, from HY5 or HFR1 to SPA1 and SPA2, to mediate their degradation.

In addition to all these different mechanisms to control the activity of the COP1/SPA complex it remains an open question if also a nuclear/cytoplasmic redistribution of SPAs, which has not been described so far, might be involved in regulation of COP1/SPA complex activity.

In contrast to COP1, SPAs are considered constitutively nuclear localized proteins (Hoecker et al., 1999; Laubinger et al., 2006) and may therefore retain COP1 in the nucleus by a light-reversible mechanism. Currently, it is still unknown if nuclear levels of COP1 or its localization in NBs in dark is altered in a *spa* quadruple mutant. Thus, it might be that SPA proteins are required to trap COP1 in the nucleus and that SPA proteins regulate COP1 nucleocytoplasmic partitioning in response to specific light conditions.

2.7 ROLES OF SPA PROTEINS IN OTHER LIGHT-REGULATED PROCESSES

In order to induce photomorphonecic development in response to light, photoactivated phytochromes need to repress the activity of two main negative regulators involved in this process: COP1/SPA complex and PIFs.

In light phytochromes bind to PIFs and induce their phosphorylation by an as yet unknown kinase. Afterward the phosphorylated form of PIF is ubiquitylated by various E3 ligases and degraded through the 26S proteasome pathway to initiate photomorphogenesis (Xu et al., 2015).

An increasing number of works provided evidence that in phytochromes signaling the COP1/SPA and PIFs regulatory pathways are not strictly separated.

In particular, the protein degradation of HFR1, which negatively regulates PIFs (PIF1, PIF4 and PIF5) activity by forming inactive HFR1/PIF dimers, is mediated by the COP1/SPA complex (Yang et al., 2005; Yang et al., 2005; Lorrain et al., 2009). This enhanced activity of PIFs, caused by COP1-dependent degradation of HFR1, is

particularly important for the control of shade avoidance responses. Thus, in shade avoidance two convergent pathways are operating which involve the action of phytochromes on PIFs (Lorrain et al., 2008; Pedmale et al., 2016) and COP1 (Pacin et al., 2013; Pacin et al., 2016) (Figure 2.2).

In addition to this example of indirect regulation, it has been demonstrated that COP1 does not only regulate the protein turnover of SPA1 and SPA2 but also the degradation of PIF1 (Xu et al., 2014; Zhu et al., 2015). PIF1 also forms complexes with COP1 and SPA1. In response to short light treatments COP1 and SPAs promote rapid degradation of PIF1 (Xu et al., 2014; Zhu et al., 2015). In dark-grown seedlings PIF1 also interacts with COP1/SPA but, in contrast to light, here it enhances COP1 E3 ligase activity. This indicates that in the dark PIF1 works as a positive regulator of COP1/SPA1 function. Therefore, PIF1 may work as a ‘‘molecular clamp’’ that ties together COP1 and SPA1, and thereby stabilizes the complex (Xu et al., 2014). This mechanisms for the regulation by (and of) COP1/SPA seems to be an exclusive feature of PIF1 among the members of the PIF family.

Therefore, because COP1 regulates the turnover of PIF1, SPA2, and possibly SPA1, it is possible that light triggers a change in the substrate specificity of the COP1/ SPA complex instead of simply inactivating it. The well-established role of COP1 in the degradation of positive regulators of photomorphogenesis in the dark (Lau and Deng 2012) is, therefore, extended to degradation of negative regulators in light (Zhu et al. 2015; Chen et al. 2015). These findings further highlight the important function in repressing the COP1/SPA complex in light signaling pathway. It is widely accepted that SPA proteins function, together with COP1, for the proper control of protein stability of HY5 and other factors, such as HFR1 and CO, in phytochrome and cryptochrome downstream signaling (Yang et al., 2005; Laubinger et al., 2006; Lian et al., 2011).

Nevertheless, given that the Arabidopsis *spaQ* mutant, that lacks any functional SPAs, is viable in contrast to the strong *cop1* mutant alleles which are lethal, indicates that COP1 possesses SPA independent functions (Mcnellis et al., 1994; Laubinger et al., 2004; Ordonez-Herrera et al., 2015). Besides responses to visible light, the progresses in the last few years have expanded the role of COP1 also in other processes including UV-B signaling, plant defense, hormone signaling and cold acclimation (Lau and Deng, 2012). Regulating the stability of its target HY5, which has been identified to play a central role

in hormone (i.e. gibberellins and cytokinine) and cold acclimation responses, COP1 has been proposed as integrator hub of environmental and hormone signaling (Alabadi and Blazquez, 2008; Catala et al., 2011; Lau and Deng, 2012). In addition, HY5 transcription factor also mediates abscisic acid (ABA) responses during seed germination, early seedling growth and root development in Arabidopsis. Recently it has been identified that salt stress, which promotes the retention of COP1 in the cytosol, and ethylene, which enhances the movement of COP1 into the nucleus, antagonistically regulate the nucleocytoplasmic partitioning of COP1 (Yu et al., 2016), which affects the accumulation of HY5 protein and subsequently resulting in the transcriptional repression of *ABSCISIC ACID* (*ABA) INSENSITIVE 5 (ABI5)* a major mediator of plant ABA responses during seed germination (Chen and Xiong, 2008; Yu et al., 2013; Yu et al., 2016).
In addition, it is well established that photomorphogenic responses induced by UV-B light, which depend on the photoreceptor UVR8 and its downstream component COP1, does not require SPA proteins (Oravecz et al., 2006). Thus, it is still questionable if SPAs play a role in the regulation of COP1's E3 ubiquitin ligase activity in response to stimuli other than visible light.

2.8 SPAS MIGHT BE REQUIRED TO ACHIEVE WAVELENGTH SPECIFICITY IN VISIBLE LIGHT

Genome-wide analysis, during early stages of Arabidopsis seedling de-etiolation, showed that only UV-B light regulates a unique set of genes, whereas a limited number of genes are regulated by specific wavelengths of the visible spectra (Peschke and Kretsch, 2011). This may reflect that phytochrome and cryptochrome downstream signaling pathways converge on the COP1/SPA complex, whereas UVR8 uses a distinct pathway not depending on SPAs (Oravecz et al., 2006). Nevertheless, there are a number of genes that is regulated by specific wavelengths in the visible range of the light spectrum and the question is how this specificity can be achieved. The heterogeneous SPA-COP1 core complex consists of two COP1 molecules and two either identical or different SPA molecules. Despite partial redundancy between the four members of the SPA protein family in Arabidopsis, different light- and organ-specific contributions of

each *SPA* gene to distinct developmental processes are present (Zhu et al., 2008). Therefore, a wavelength-specificity could be the result of the specific binding of a particular photoreceptor (i.e cryptochromes or phytochromes) with a specific SPA member (Zhu et al., 2008; Sheerin et al., 2015) (Figure 2.3).

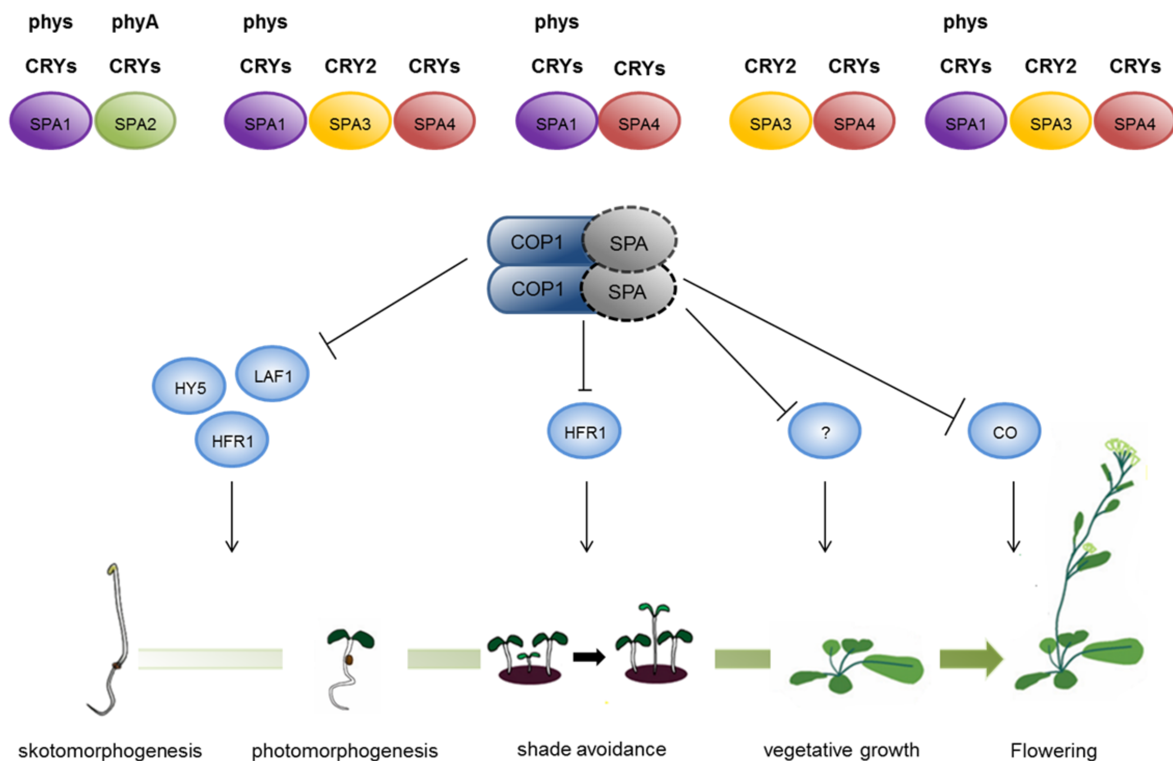

FIGURE 2.3: OVERLAPPING AND DISTINCT FUNCTIONS OF SPA PROTEINS DURING PLANT DEVELOPMENT.

Each SPA member interacts specifically with phys (phyA, phyB) and/or CRYs (CRY1, CRY2). Both SPA1 and SPA2 are involved in the inhibition of photomorphogenesis in dark-grown seedlings while SPA1, and to a lesser extent SPA3 and SPA4, promotes de-etiolation. SPA1 and SPA4 are main regulators of the shade avoidance response, SPA3 and SPA4 are important for vegetative plant growth. SPA1 is the predominant player in the suppression of flowering under short-day conditions. Moreover SPA proteins contribute differently to plant developmental processes by forming a heterogeneous SPA-COP1 core complex which could display substrate specificity to different TFs (i.e. HY5, HFR1 LAF1, CO) (see text for references).

In addition, plants might achieve a wavelength-specific regulation of the turnover of different substrates by combining COP1 with specific sets of SPA proteins.

Moreover, because the expression of the four *SPA* genes in Arabidopsis also depends on the developmental stage and the tissue (Fittinghoff et al., 2006), it suggests that

spatiotemporal control of SPA proteins would also contribute to functional specificity of the COP1/SPA complex (Figure 2.3).

Finally, the importance of the COP1/SPA complex in plants raises the question when this regulatory pathway evolutionary arose. Whereas COP1 orthologues are conserved not only in cryptograms and other seed plants species besides Arabidopsis but also in invertebrate and vertebrate animals e.g. humans (Lau and Deng, 2012) SPA proteins appear to be restricted to plants (Ranjan et al., 2014). Whole genome sequencing has shown that *SPA* genes exist in early diverged land plants, such as in Physcomitrella, (Ranjan et al., 2014). In moss, a total of nine *COP1* paralogs have been predicted in contrast to the *COP1* single copy gene present in rice and Arabidopsis.

Interestingly, the expression of Physcomitrella *COP1* with the highest sequence similarity to that of Arabidopsis COP1 has been shown to complement almost all phenotypic aspects of the Arabidopsis *cop1* mutants. This indicates that the core function of COP1 is under strong negative selection and moreover it that has been functionally conserved during evolution (Ranjan et al., 2014).

Contrary, SPA proteins exhibit considerable functional divergence. Gene duplication events preceding the evolution of monocots and dicots generated a split of the *SPA* gene lineage into two subgroups *SPA1/SPA2* and *SPA3/SPA4*. In Physcomitrella are present only two *SPA* genes; *SPAa* and *SPAb*, which display a high amino acid identity of the predicted proteins. This suggests that they possibly derive from more recent duplication event based on an ortholog of Arabidopsis SPA1/2 (Ranjan et al., 2014). Interestingly, Physcomitrella and rice SPA orthologues expressed in the Arabidopsis *spa* mutants are unable to fully restore the wild-type phenotype (Ranjan et al., 2014).

This failure of full complementation suggests that SPAs in seed plants and Physcomitrella are too different to allow substitution. It is therefore reasonable to assume that gene duplication events during the evolution have been required to have different SPAs, which differ in terms of i.e. substrate specificity and regulation by different receptors, in order to achieve a huge diversity of SPA function in the different organisms. For this reason it seems likely that COP1, as evolutionary conserved, functions as a core of the COP1/SPA complex and that SPA proteins confer specificity and might have evolved to place COP1 activity under light control.

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LIST OF PUBLICATIONS AND MANUSCRIPTS

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Light-Activated Phytochrome A and B Interact with Members of the SPA Family to Promote Photomorphogenesis in Arabidopsis by Reorganizing the COP1/SPA Complex

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Phytochromes function as red/far-red photoreceptors in plants and are essential for light-regulated growth and development. Photomorphogenesis, the developmental program in light, is the default program in seed plants. In dark-grown seedlings, photomorphogenic growth is suppressed by the action of the CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1)/SUPPRESSOR OF *phyA-105* (SPA) complex, which targets positive regulators of photomorphogenic growth for degradation by the proteasome. Phytochromes inhibit the COP1/SPA complex, leading to the accumulation of transcription factors promoting photomorphogenesis; yet, the mechanism by which they inactivate COP1/SPA is still unknown. Here, we show that light-activated phytochrome A (phyA) and phytochrome B (phyB) interact with SPA1 and other SPA proteins. Fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy analyses show that SPAs and phytochromes colocalize and interact in nuclear bodies. Furthermore, light-activated phyA and phyB disrupt the interaction between COP1 and SPAs, resulting in reorganization of the COP1/SPA complex in planta. The light-induced stabilization of HFR1, a photomorphogenic factor targeted for degradation by COP1/SPA, correlates temporally with the accumulation of phyA in the nucleus and localization of phyA to nuclear bodies. Overall, these data provide a molecular mechanism for the inactivation of the COP1/SPA complex by phyA- and phyB-mediated light perception.

INTRODUCTION

Plants use light not only for photosynthesis but also as a source of information, which is important to adapt growth and development to ever-changing and often hostile environments. For light perception, plants possess several classes of photoreceptors. The cryptochromes (CRY1 and CRY2), phototropins (PHOT1 and PHOT2), and ZEITLUPE family proteins are receptors for blue, UVR8 for UV-B, and phytochromes for red (R) and far-red (FR) light (Kami et al., 2010; Rizzini et al., 2011). Phytochromes exist in two states, the inactive Pr and the biologically active Pfr form that maximally absorb in R and FR, respectively (Mancinelli, 1994). By absorption of light, phytochromes reversibly convert between the two forms, resulting in wavelength-specific Pfr:Ptot (Ptot = Pr + Pfr) ratios. The phytochrome gene family in the model plant *Arabidopsis thaliana* consists of five members, of which phyA and phyB play a dominant role (Franklin and Quail, 2010). PhyB, the major phytochrome species in light-grown and adult plants, is important for responses to R light and for measuring the R:FR ratio (Kami et al., 2010; Li et al., 2011). By contrast, phyA is highly abundant in dark-grown plants but rapidly degraded in light (Li et al., 2011). Responses induced by low Pfr:Ptot ratios, which are typically established by continuous irradiation with FR or light pulses of any wavelength, depend on phyA (Kami et al., 2010). As such, phyA is required for seedling establishment in light environments dominated by FR light, for instance, the undergrowth of forests (Yanovsky et al., 1995).

Depending on the light conditions, plants follow different developmental programs after germination: skotomorphogenesis in the dark and photomorphogenesis in light. The default developmental program in seed plants is photomorphogenesis, which is repressed in the absence of light. PHYTOCHROME INTERACTING FACTORs (PIFs), CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), and members of the SUPPRESSOR OF *phyA-105* (SPA) family, are crucial to inhibit photomorphogenic growth in the dark (Deng et al., 1991; Laubinger et al., 2004; Leivar et al., 2008; Shin et al., 2009). The E3 ubiquitin ligase COP1 and SPA proteins form oligomeric complexes, which target positive regulators of photomorphogenesis for degradation by the proteasome (Seo et al., 2003; Jang et al., 2005; Zhu et al., 2008). The SPA proteins (SPA1-SPA4 in Arabidopsis) are required for the E3 ubiquitin ligase function of the

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COP1/SPA complex and may play a role in recognition of substrates, including LONG HYPOCOTYL IN FAR-RED1 (HFR1) and LONG AFTER FAR-RED1 (LAF1) (Yang and Wang, 2006). Moreover, PIFs also form complexes with COP1 and SPA1 and enhance the substrate recruitment, autoubiquitination, and transubiquitination activity of COP1 (Xu et al., 2014). Upon activation by light, phytochromes translocate from the cytosol into the nucleus, where they trigger photomorphogenic responses by both inhibiting binding of PIFs to their target promoters and targeting them for degradation and by stabilizing the targets of the COP1/SPA complex (Kami et al., 2010; Park et al., 2012). Inactivation of COP1/SPA by phytochromes has been proposed, allowing accumulation of transcription factors, such as ELONGATED HYPOCOTYL5 (HY5), HFR1, and LAF1, which trigger

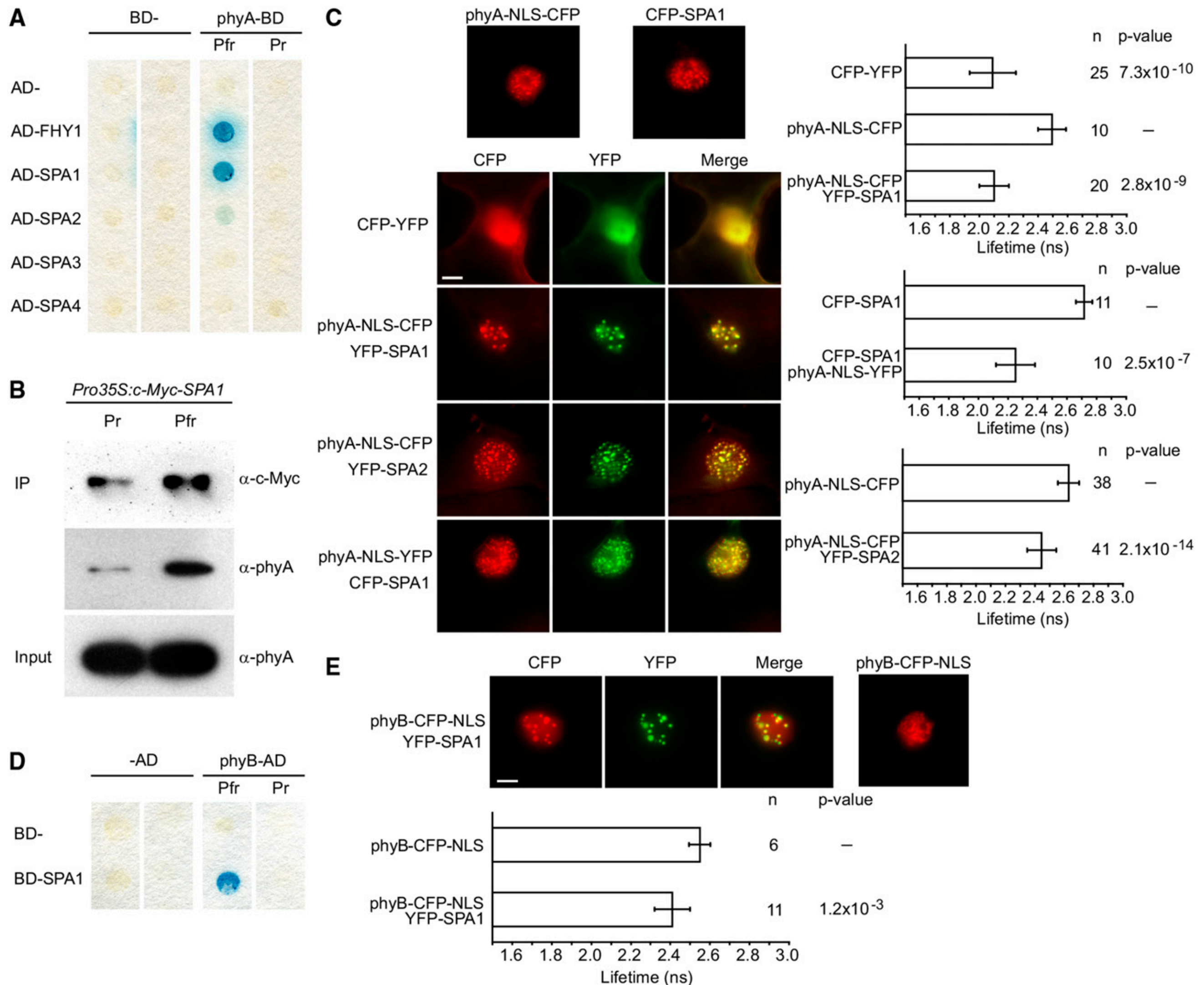

Figure 1. Light-Activated phyA and phyB Interact with SPAs in Nuclear Bodies.

(A) Yeast two-hybrid protein-protein interaction assay. The phyA-GAL4-DNA binding domain (phyA-BD) fusion was coexpressed with GAL4-activation domain (AD-) fusions of FHY1 and SPA1-4. Yeast cells were lifted from chromophore-supplemented plates that had been incubated for 48 h under either constant R (Pfr) or FR light (Pr). Interaction was detected by an X-Gal filter lift assay.

(B) Coimmunoprecipitation of phyA with SPA1. SPA1 was immunoprecipitated from stable transformed Arabidopsis plants expressing c-Myc-tagged SPA1 using an α-c-Myc antibody. Plants were grown in darkness (Pr) or darkness followed by a 5-min R light pulse (Pfr) prior to immunoprecipitation. α-phyA antibodies were used to detect phyA copurifying with c-Myc-SPA1.

(C) FRET-FLIM analysis of NB-localized phyA and SPA1/SPA2 CFP and YFP fusions transiently expressed under the control of the 35S promoter in *N. benthamiana*. Left, epifluorescent microscope visualization of subcellular localization of phyA, SPA1, and SPA2 upon transfer from darkness to light; right, fluorescence lifetime of the donor (CFP). Error bars show one SD. n = number of measurements. P values indicate t test analysis for statistically significant differences. Bar = 10 μm.

(D) Yeast two-hybrid protein-protein interaction assay as for **(A)** except BD-SPA1 and phyB-AD fusions were used to avoid phyB autoactivation.

(E) FRET-FLIM analysis as for **(C)** of NB-localized phyB-CFP and YFP-SPA1.

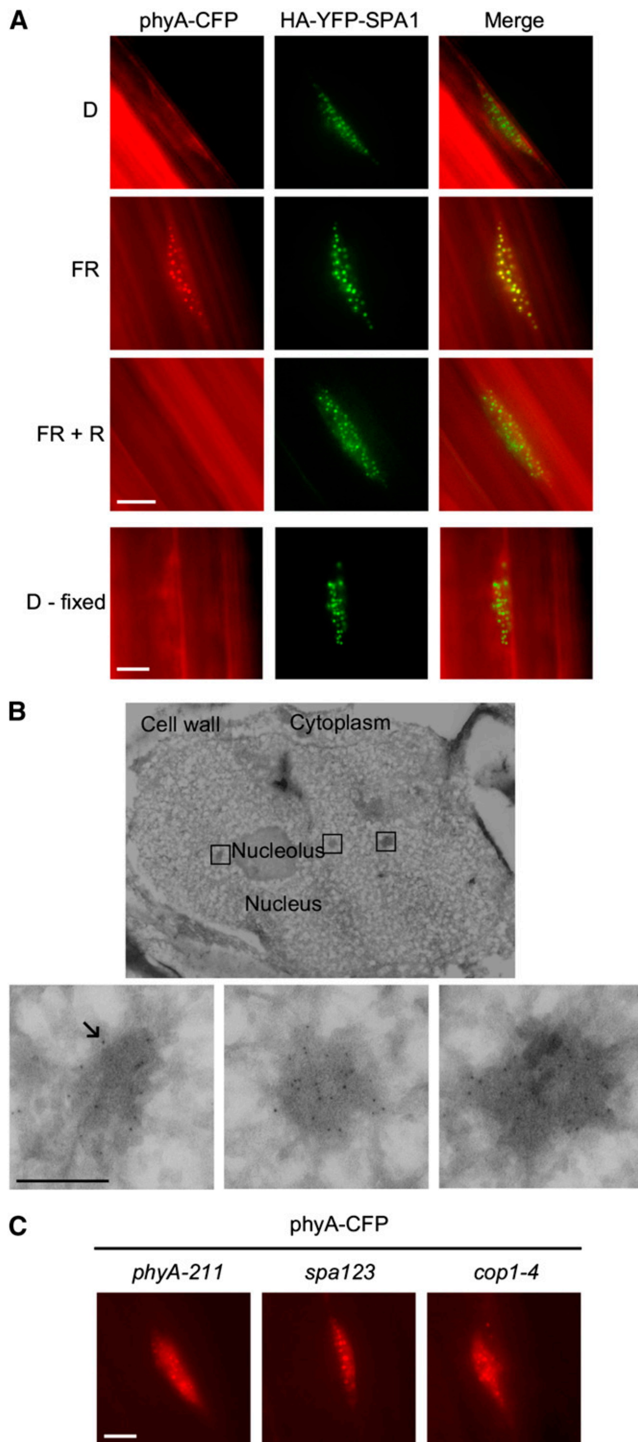

Figure 2. phyA Colocalizes with SPA1 in Arabidopsis Nuclear Bodies.

(A) Epifluorescence microscopy visualization of phyA-CFP and HA-YFP-SPA1 in hypocotyl cells of stable cotransformed *ProPHYA:PHYA-CFP* and *Pro35S:HA-YFP-SPA1* Arabidopsis plants. Seedlings were grown for 4 d in darkness and treated with either no light (D), 6 h FR light, or 6 h FR followed by 6 h R light (FR + R). Additionally, 4-d-old etiolated seedlings were fixed with formaldehyde prior to microscopy (D - fixed).

(B) Immunoelectron microscopy localization of phyA in wild-type Arabidopsis Col-0 hypocotyl nuclei. Seedlings were grown in darkness for 4 d and treated with 6 h FR light followed by 5 min R light prior to fixation. Endogenous phyA was probed with α-phyA antibodies and detected with protein A-labeled 6-nm gold particles (indicated by arrow). Upper panel: overview (nucleus). Lower panels: enlarged areas (nuclear bodies). Bar = 200 nm.

(C) Epifluorescence microscopy visualization of phyA-CFP expressed from *ProPHYA:PHYA-CFP* in *phyA-211*, *spa1-7 spa2-1 spa3-1* (*spa123*), and *cop1-4* Arabidopsis backgrounds. Seedlings were grown in darkness for 4 d, followed by 6 h FR. Bars in **(A)** and **(C)** = 4 μm.

photomorphogenic development (Kami et al., 2010; Li et al., 2011). Although it is established that phytochromes must inactivate the COP1/SPA complex in light-grown seedlings, the molecular mechanism is still unknown. Here, we show that both light-activated phyA and phyB compete with COP1 for binding to SPA1 and other SPA proteins, suggesting that phytochromes promote photomorphogenesis by inhibiting the direct interaction of COP1 and SPAs, leading to the inactivation of the COP1/SPA complex. A similar mechanism has been proposed for the CRY1-mediated inactivation of COP1/SPA, while CRY2 appears to employ a different method of inhibition that does not disrupt the direct interaction of COP1 and SPA1 (Lian et al., 2011; Liu et al., 2011; Zuo et al., 2011).

Many components involved in light signaling, including phytochromes, COP1, SPAs, and PIFs, form nuclear bodies (NBs) (Van Buskirk et al., 2012). The ability to form NBs often correlates with the physiological activity or degradation of these components, suggesting that NBs are critical for signal transduction and protein turnover. In this article, we show that SPA1 localization to NBs is not dependent on light, in contrast to phyA, and that the phyA G727E and phyA E777K mutants, which are impaired in NB localization, do not interact with SPA1. Yet, SPA proteins appear not to be essential for recruiting phyA into NBs

Light-induced exclusion of COP1 from the nucleus has been proposed as a mechanism to explain inactivation of COP1 in light (Osterlund and Deng, 1998). However, we show that FR-induced accumulation of HFR1, a target of COP1/SPA E3 ubiquitin ligase activity (Jang et al., 2005), precedes nuclear export of COP1 and that HFR1 is stabilized by FR light treatments that temporally correlate with phyA nuclear accumulation and NB localization. This is consistent with a mechanistic model in which disruption of the direct interaction of COP1 and SPAs by light-activated phyA is sufficient to prevent COP1/SPA-induced degradation of HFR1 independent of the dissociation of the COP1/SPA complex and COP1 relocation to the cytosol.

RESULTS

Light-Activated Phytochromes Interact with SPAs in NBs

We used yeast two-hybrid screening to identify proteins that directly interact with phyA. In contrast to previous screens involving phytochromes (Ni et al., 1998), we incorporated chromophore to produce photoactive phyA. Yeast is unable to synthesize the

naturally occurring chromophore of seed plant phytochromes, phytochromobilin (PΦB). However, previous reports indicate that phycocyanobilin (PCB) extracted from cyanobacteria can substitute in vivo (Kami et al., 2004). Screening on media supplemented with PCB, we identified SPA1 as a phyA-interacting protein (Figure 1A). In a similar fashion to the known phyA interactor FHY1 (Hiltbrunner et al., 2005), SPA1 preferentially bound to the active Pfr form of phyA, with no detectable interaction with the inactive phyA Pr. Supporting this observation, coimmunoprecipitation using Arabidopsis expressing c-MYC-SPA1 also demonstrated a light-dependent pull-down of phyA with SPA1 (Figure 1B). phyA has previously been shown to coimmunoprecipitate with SPA1 from FR-grown Arabidopsis seedlings; however, a direct interaction was never shown (Saijo et al., 2008), and copurification of phyA and SPA1 has been attributed to shared interaction with COP1.

To establish if phyA and SPA1 interact in planta, we coexpressed cyan and yellow fluorescent protein (CFP and YFP) fusions of either SPA1 or phyA in *Nicotiana benthamiana* (wild tobacco) under the control of the strong 35S mosaic virus promoter. As phyA nuclear import was rather inefficient in *N. benthamiana* leaves, a nuclear localization signal (NLS) was included for phyA fusions to ensure sufficient phyA in leaf epidermal cell nuclei (Genoud et al., 2008; Supplemental Figure 1). phyA was subsequently activated through exposure to epifluorescent light. In *N. benthamiana* leaves transiently coexpressing YFP-SPA1 and phyA-NLS-CFP (or CFP-SPA1 and phyA-NLS-YFP), the two proteins colocalized to NBs (Figure 1C). Furthermore, in fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) analyses of NB-localized phyA and SPA1 (phyA-NLS-CFP/YFP-SPA1 or CFP-SPA1/phyA-NLS-YFP), the fluorescence lifetime of the donor (CFP) was strongly reduced as compared with negative controls, supporting an interaction of SPA1 and phyA within NBs (Figure 1C).

We also observed colocalization of phyA-NLS-CFP with YFP-SPA2, -SPA3, and -SPA4 in NBs of *Agrobacterium tumefaciens*-infiltrated *N. benthamiana* leaves, though only for SPA2 was a significant reduction in fluorescence lifetime detected (Figure 1C; Supplemental Figure 1). Yeast two-hybrid assays also detected binding of phyA to SPA2, but not SPA3 or SPA4, though weak interactions cannot be excluded (Figure 1A; Supplemental Figure 2). Thus, a positive signal in FRET-FLIM analyses was specifically observed for SPA proteins that physically interacted with phyA but not for those that only colocalized with phyA in NBs.

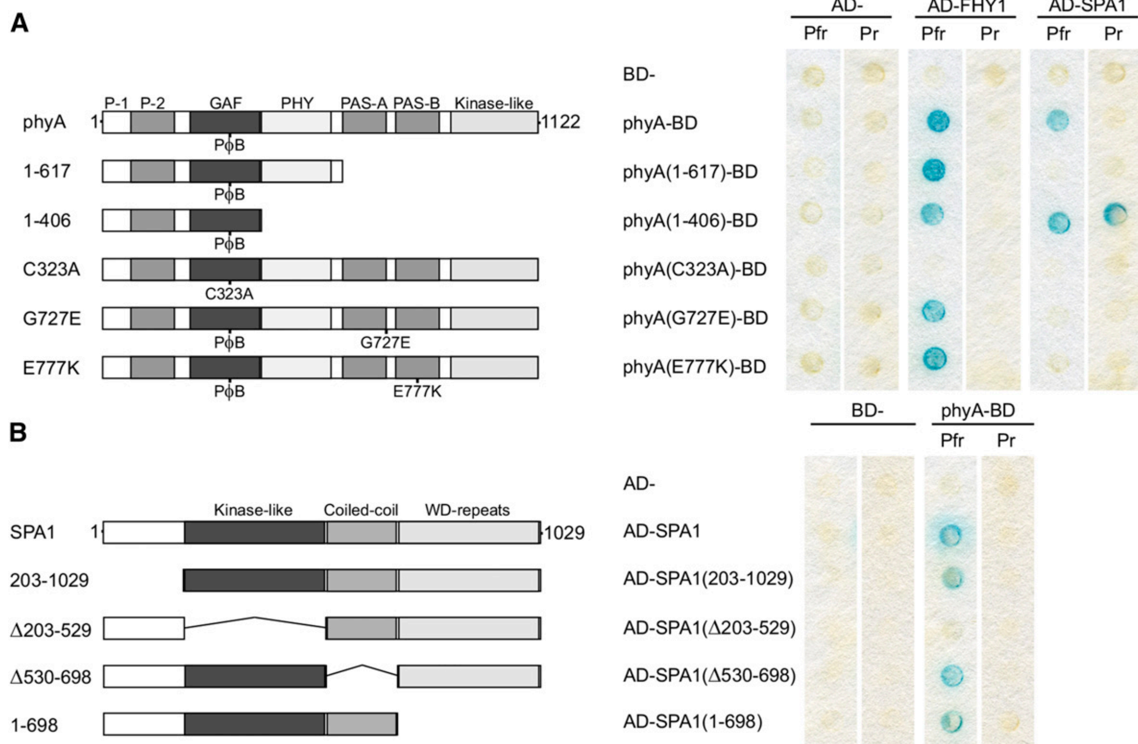

Figure 3. The N Terminus of phyA Interacts with the Kinase-Like Domain of SPA1.

(A) Domain and mutant analysis of phyA. phyA truncations and amino acid substitutions fused to the GAL4-DNA binding domain (BD) were coexpressed with GAL4-activation domain (AD)-SPA1. Yeast cells were lifted from chromophore-containing plates that had been incubated for 48 h under either constant R (Pfr) or FR (Pr) light. Interaction was detected by an X-Gal filter lift assay. Left, schematic of the phyA truncations and substitutions; right, X-Gal filter lift assay.

(B) Domain analysis of SPA1. SPA1 truncations or deletions fused to the GAL4 AD were coexpressed with phyA-BD. The yeast two hybrid assay was performed as described in **(A)**. Left, schematic of SPA1 truncations and deletions; right, X-Gal filter lift assay.

Immunoblot analysis of phyA and SPA1 protein levels and quantitative assays are shown in Supplemental Figure 5.

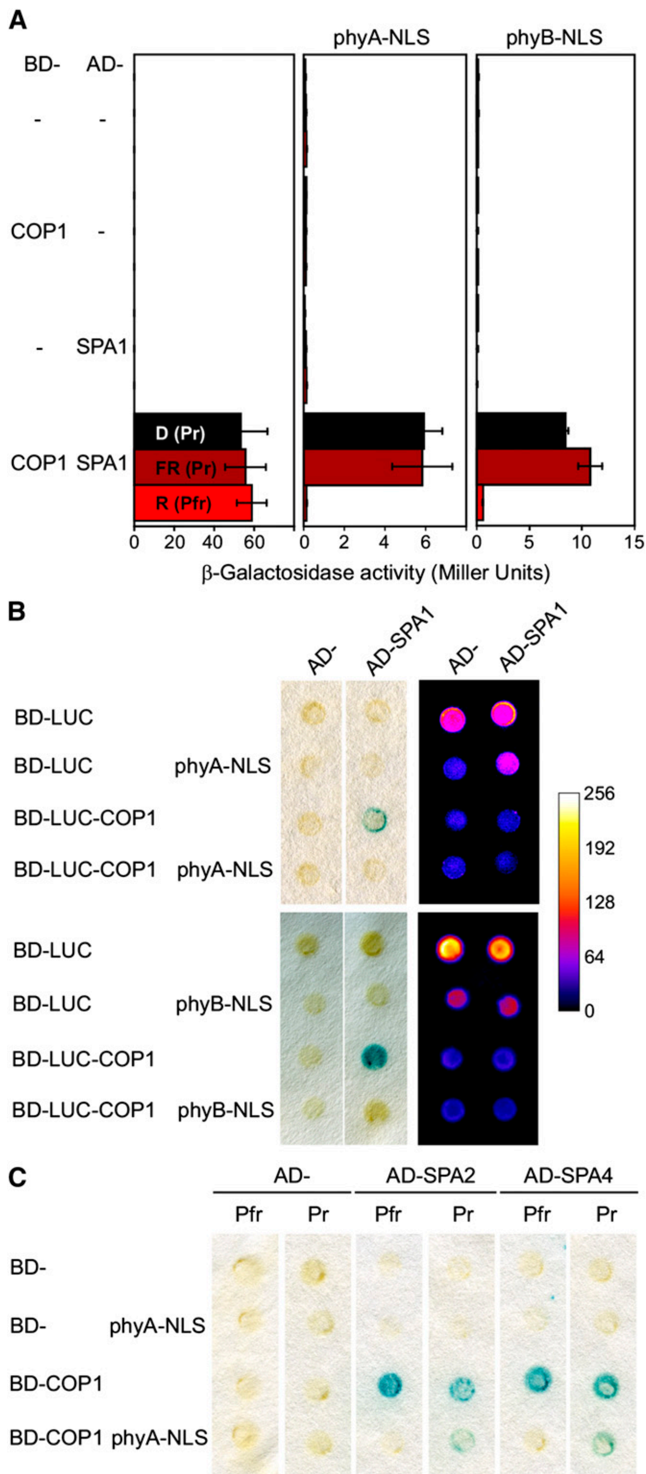

Figure 4. Phytochromes Inhibit the Interaction of COP1 with SPA Proteins.

(A) Yeast three-hybrid analysis of the effects of phyA and phyB on the COP1-SPA1 interaction. COP1 and SPA1 were expressed as a standard yeast two-hybrid protein-protein interaction pair (BD-COP1 and AD-SPA1). Phytochromes were coexpressed as additional proteins, with C-terminal nuclear localization signals (phyA/B-NLS). Yeast cells were grown on chromophore-supplemented plates for 72 h under either constant darkness (D), R, or FR light, and the interaction of COP1 and SPA1 detected using ONPG. Values are the average of nine assays; error bars display 1 SD.

(B) Yeast three-hybrid analysis, including a LUC fusion of COP1 (BD-LUC-COP1) and AD-SPA1, performed as in **(A)** under constant R light. Left, X-Gal filter lift assays; right, in vivo luciferase activity. Plates were sprayed with 5 mM luciferin and imaged using a CCD camera. Arbitrary light signal intensity is indicated in the adjacent scale.

(C) Yeast three-hybrid analysis of the effects of phyA on the COP1-SPA2/4 interaction. Performed as for **(A)** in either darkness (Pr) or constant R light (Pfr), and interaction was detected by X-Gal filter lift assay. Immunoblot analysis of BD-LUC-COP1, AD-SPA1, and phyA-NLS protein levels is shown in Supplemental Figure 6.

The major Arabidopsis phytochrome in adult plants, phyB, has been reported to interact with SPA1 in a light-independent manner (Zheng et al., 2013). Using altered conditions to avoid autoactivation, and to include full-length photoactive phytochrome, a light-dependent interaction between SPA1 and phyB was observed in yeast two-hybrid assays, suggesting that both phyA and phyB may share a similar light-dependent interaction with SPA1 (Figure 1D; Supplemental Figure 2). phyB was also observed to colocalize as a CFP-NLS fusion with YFP-SPA1 in NBs (Figure 1E). Furthermore, a significant decrease in donor fluorescence life time was measured in FRET-FLIM analyses, suggesting that phyB and SPA1 also interact in NBs.

SPA1 NBs Do Not Depend on Light

Using stable transgenic *ProPHYA:PHYA-CFP* and *Pro35S:HA-YFP-SPA1* Arabidopsis lines, we confirmed the colocalization of phyA and SPA1 in NBs of seedlings exposed to FR (Figure 2A; Supplemental Figure 3A). Furthermore, we performed immunofluorescence and immunoelectron microscopy to show that native Arabidopsis phyA is localized to NBs after irradiation with light and that phyA-containing NBs correspond to electron-dense structures of 100 to 200 nm in diameter (Figure 2B; Supplemental Figure 4). NBs are therefore not an artifact of transgene expression, consistent with similar experiments performed on pea (*Pisum sativum*) phyA (Hisada et al., 2000, 2001). As *SPA1* is normally a light-induced gene, we used constitutive 35S promoter-driven expression to investigate the dependence of light on protein localization (Hoecker et al., 1999; Fittinghoff et al., 2006). In contrast to phyA, SPA1 was also present in NBs of dark-grown, etiolated seedlings (Figure 2A; Supplemental Figure 3B). To exclude the possibility that brief light exposure during sample preparation or image acquisition induced the formation of SPA1-containing NBs, we fixed the seedlings with formaldehyde under green light (525 nm) prior to microscopy. Even in dark-grown seedlings expressing HA-YFP-SPA1 fixed with formaldehyde, we observed SPA1 in NBs (Figure 2A). Thus, SPA1 NB-localization does not depend on light, which is in contrast to other components involved in light signaling (phytochromes, cryptochromes, and PIFs), suggesting that SPA proteins could function in recruitment of phyA into NBs (Van Buskirk et al., 2012). To test the requirement of SPA proteins for phyA NB

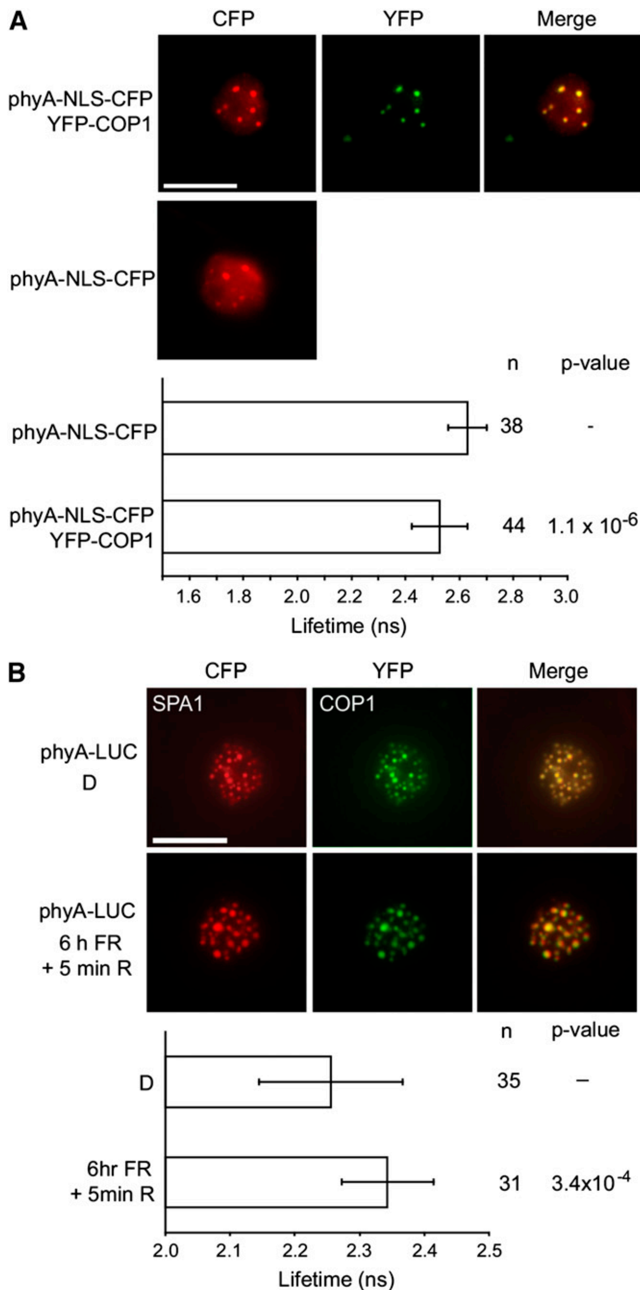

Figure 5. Reorganization of the COP1/SPA1 Complex by phyA upon Irradiation with Light.

(A) FRET-FLIM analysis of NB-localized phyA and COP1 CFP and YFP fusions in transiently transformed *N. benthamiana* plants. Upper panels show epifluorescent microscope visualization of subcellular localization. The lower panel displays the fluorescence lifetime of the donor (CFP).

(B) FRET-FLIM analysis of the disruption of the interaction between COP1 and SPA1. CFP-SPA1, YFP-COP1, and phyA-LUC were co-transformed into *N. benthamiana*. Plants were grown in darkness (D) or darkness followed by 6 h FR and a 5-min R pulse to activate phyA nuclear transport and NB formation. Upper panels show epifluorescent microscope visualization of subcellular localization. The lower panel displays the fluorescence lifetime of the donor (CFP).

Error bars show 1 SD. *n* = number of measurements. P values indicate *t* test analysis for statistically significant differences. Expression of constructs in **(A)** and **(B)** was driven by the 35S promoter. Bars = 10 μm.

formation, we transformed *ProPHYA:PHYA-CFP* into a *spa123* triple mutant background (Figure 2C). Interestingly, phyA-CFP localization was unaffected. Together with the previous observation that SPA4 only plays a minor role in dark-grown seedlings (Laubinger et al., 2004), this leads to the conclusion that another as yet uncharacterized factor is likely sufficient for NB formation. COP1 is also localized to NBs in darkness, so we also investigated phyA-CFP localization in a *cop1-4* mutant background. Similar to the *spa123* background, phyA-CFP still localized to NBs after activating light exposure in a *cop1-4* background (Figure 2C). However, *cop1-4* is a weak COP1 mutation that expresses a truncated COP1 protein (complete loss-of-function alleles are lethal), and it cannot be concluded for certain that COP1 is not involved in phyA NB localization (McNellis et al., 1994).

The Photosensory Domain Is Not Sufficient for the Pr/Pfr Specificity of the phyA-SPA1 Interaction

Consistent with the Pfr-dependent interaction of phyA and SPA1, we found that the phyA C323A mutant, which cannot covalently bind chromophore (Rockwell et al., 2006), does not interact with SPA1 (Figure 3A). Unexpectedly, expression of an N-terminal fragment of phyA (1 to 406) resulted in a light-independent interaction with SPA1, yet a larger fragment including the PHY domain (1 to 617) resulted in a loss of binding, despite retaining a light-dependent interaction with FHY1 (Figure 3A; Supplemental Figure 5). These results indicate a binding site located in the N-terminal 406 residues of phyA and that access is regulated through residues 407 to 1122, including the PHY domain, which is expected to form contacts with the chromophore and GAF domain (Essen et al., 2008; Yang et al., 2009).

The *phyA-103* and *phyA-302* mutants are insensitive to FR light due to missense mutations in the PAS-A (G727E) or PAS-B (E777K) domains of phyA, respectively (Dehesh et al., 1993; Yanovsky et al., 2002). As neither phyA mutant is recruited into NBs, where we observed the phyA-SPA1 interaction in planta, we investigated if phyA G727E or phyA E777K are altered in their interaction with SPA1. While these mutations did not affect the interaction with FHY1 in yeast, they both abolished detectable binding of SPA1 (Figure 3A). As both mutations are located in the C terminus of phyA, they are likely involved in the aforementioned C terminus-mediated light dependency of the SPA1-phyA interaction, rather than residues that directly bind SPA1. As phyA still forms NBs in the *spa123* mutant background, it seems likely that other uncharacterized phyA interactions also are affected in phyA G727E and phyA E777K, leading to loss of NB localization.

The Kinase-Like Domain of SPA1 Is Essential for the Interaction with phyA

SPA proteins consist of a variable N terminus and three conserved domains: a kinase-like domain, a coiled-coil domain, and

a C-terminal WD-repeat domain. Using yeast two-hybrid assays, we found that the kinase-like domain of SPA1 is essential for binding to phyA (Figure 3B; Supplemental Figure 5). By contrast, the N-terminal extension, the coiled-coil, and WD-repeat domains are not essential for the interaction with phyA, although they may contribute to the strength of the interaction.

Phytochromes Inhibit the Interaction of SPAs and COP1

It is well established that phyA inhibits the COP1/SPA-dependent turnover of transcription factors, such as HY5, HFR1, LAF1, and CO, but the molecular mechanism has not been described (Ang et al., 1998; Seo et al., 2003; Duek et al., 2004; Jang et al., 2005; Yang et al., 2005). Using yeast three-hybrid assays, we investigated if light-activated phyA is able to regulate the interaction of COP1 and SPA proteins. Under activating light conditions, coexpression of phyA inhibited the interaction of COP1 with SPA1, whereas only weak inhibition was observed in the dark, even though the phyA protein levels were lower in light than in dark-grown yeast cells (Figures 4A and 4B; Supplemental Figure 6). Neither COP1 nor SPA1 protein abundance was affected by the presence of phyA in yeast, eliminating regulation of protein stability as a possible explanation. In addition, similar photoactivated phyA-dependent inhibition of the interaction between COP1 and both SPA2 and SPA4 was observed, indicating that phyA can bind and inhibit SPA1, SPA2, and SPA4 protein in a light-dependent manner (Figure 4C). Light-activated phyB was also observed to inhibit the interaction of COP1 and SPA1 in yeast three-hybrid assays (Figures 4A and 4B).

Based on yeast three-hybrid data (Figure 4), COP1 would be expected to be excluded from NBs under FR light conditions where phyA is recruited into NBs. However, phyA has also been indicated to interact with COP1 (Seo et al., 2004; Viczián et al., 2012), suggesting that phyA-induced inactivation of the COP1/SPA complex does not require dissociation of COP1 from NBs in FR. Using FRET-FLIM measurements of phyA and COP1 expressed in *N. benthamiana*, we confirmed that light-activated phyA was associated with COP1 in planta within NBs (Figure 5A). To investigate if the interaction of COP1 and SPA1 is altered by phyA in planta, we coexpressed CFP-COP1 and YFP-SPA1 as a FRET pair in *N. benthamiana*, with the addition of a firefly luciferase (LUC) fusion of PHYA (PHYA-LUC). Following activating light conditions (6 h FR followed by 5 min R light) the fluorescence lifetime of the CFP donor was increased in FRET-FLIM measurements, consistent with disruption of the direct interaction of the COP1 and SPA1 molecules within the complex (Figure 5B; Supplemental Figure 7). To ensure that irradiation during FRET-FLIM measurement did not affect the complex, samples were also fixed following light treatments, obtaining similar results (Supplemental Figure 7).

To further characterize phyA-dependent inactivation of COP1/SPA activity in plants, we measured the timing of stabilization by light of the positive photomorphogenic factor HFR1, which is a target of the COP1/SPA complex (Jang et al., 2005). Localization of phyA-YFP to the nucleus began within minutes of FR exposure and formation of late NBs began after 2 to 4 h FR light exposure, with localization at a maximum after 6 h (Figure 6A). Using stably transformed *hfr1-4*, *Pro35S:LUC-HFR1* lines, we

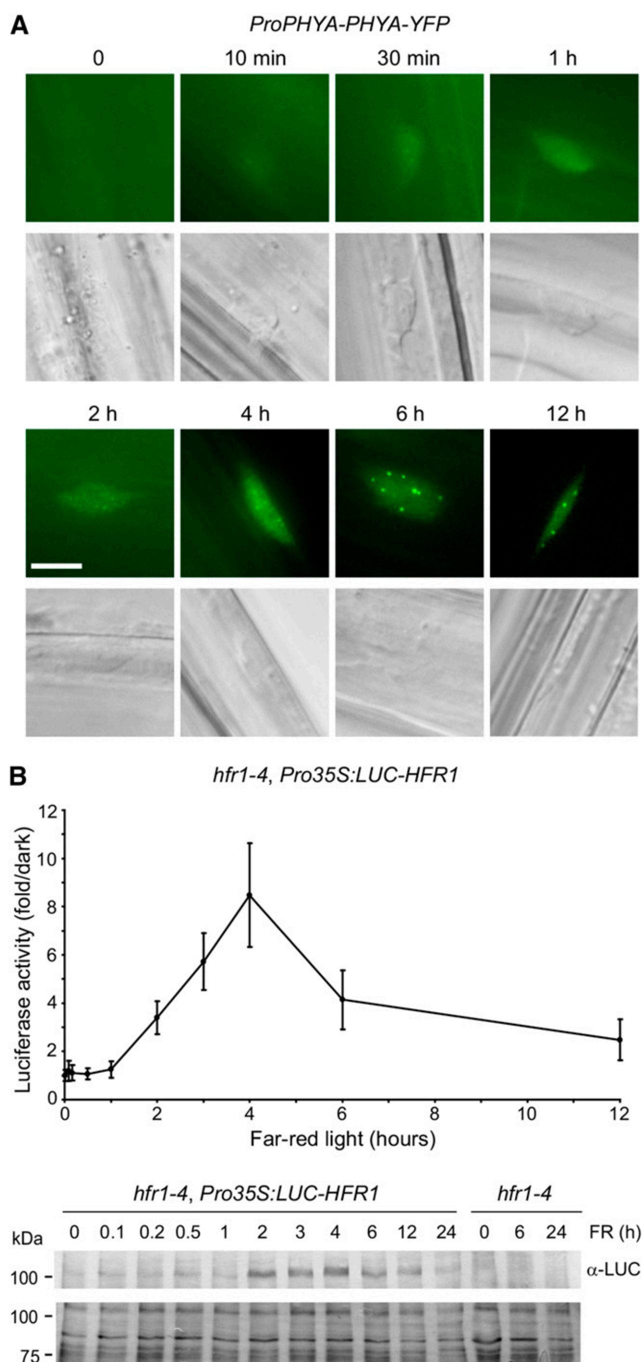

Figure 6. Temporal Correlation of HFR1 Accumulation and phyA Nuclear Localization.

(A) Time course of phyA nuclear accumulation and localization to nuclear bodies. phyA-YFP localization was observed in dark-grown *phyA-211 ProPHYA:PHYA-YFP* seedlings exposed to various lengths of FR light (10 μmol m^{-2} s^{-1}). Bar = 5 μm.

(B) HFR1 accumulates under FR light. The luciferase activity of stable transformed *hfr1-4*, *Pro35S:LUC-HFR1* Arabidopsis plants was measured in dark-grown seedlings exposed to various lengths of FR light (10 μmol m^{-2} s^{-1}). Below, immunoblot detection of LUC-HFR1 in 5 μg plant extracts. Lower panel shows amido black-stained membrane as a loading control.

quantified the protein abundance over the length of FR exposure by measuring luciferase activity and by immunoblots (Figure 6B; Supplemental Figure 8). Stabilization began after 1 h, reaching a peak at 4 to 6 h, similar to phyA nuclear accumulation and localization to NBs, consistent with these events being linked. Longer exposures to FR resulted in destabilization of HFR1, potentially by activation of an unknown feed-back mechanism.

Thus, it appears probable that in planta light-induced binding of phyA to SPA proteins can disrupt the direct interaction of COP1 and SPAs and thus inactivate the COP1/SPA complex, resulting in rapid accumulation of transcription factors initiating photomorphogenic development. COP1 and SPAs may be retained in complex through independent interactions with phyA, yet the direct interaction of COP1 and SPAs would remain disrupted in plants exposed to light (Figure 7). Interaction of phyA and SPAs may induce additional events, such as modification of COP1 and SPAs or regulation of SPA protein stability, leading to sustained downregulation of COP1/SPA activity in light-grown plants.

DISCUSSION

SPA proteins, which are represented in Arabidopsis by SPA1-4, have important functions in regulating photomorphogenesis (Hoecker et al., 1999; Laubinger et al., 2004). SPA1 and SPA2 have been shown to be the primary SPAs involved in repression of photomorphogenesis at the seedling stage, while SPA3 and SPA4 have roles during adult plant development (Laubinger and Hoecker, 2003; Laubinger et al., 2004). Here, we have shown that phyA preferentially binds to SPA1 and SPA2 and these interactions correlate well with the primary function of phyA during seedling development and lesser effects on adult plant growth (Laubinger et al., 2004). Though SPA1 is dispensable for most of adult plant development, it is required for proper control of flowering (Laubinger et al., 2006). Therefore, interaction of SPA1 and phyA is also consistent with the role of phyA in regulation of flowering in short-day conditions with FR extension (Laubinger et al., 2006). In addition, we also observed Pfr-dependent binding of SPA1 to phyB, which also plays a role in adult plants.

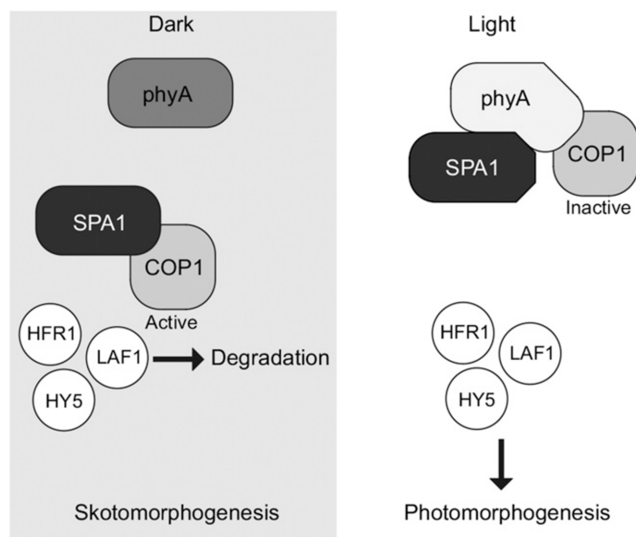

Figure 7. A Model for Light-Dependent Induction of Photomorphogenesis.

In darkness, SPA1 and other SPAs bind and activate the E3 ubiquitin ligase COP1. Activated COP1 is able to target positive photomorphogenic factors, including HFR1, LAF1, and HY5, for degradation by the proteasome. Thus, the skotomorphogenic program is established, resulting in hypocotyl elongation and repression of cotyledon development. Light-activated phyA (and phyB) can bind SPA1 and other SPAs and disrupt the direct COP1-SPA interaction. COP1, lacking a direct activating interaction with SPA, can no longer target photomorphogenic factors for degradation. These factors accumulate and promote the photomorphogenic program, inhibiting hypocotyl elongation and promoting cotyledon and leaf development. Through the direct interaction with phyA, COP1 can remain in complex even under conditions that promote photomorphogenesis.

SPAs form a light-independent complex with COP1 (Hoecker and Quail, 2001). This complex has been shown to both enhance the E3 ubiquitin ligase activity of COP1 and to bind and target for degradation many transcription factors that promote photomorphogenesis, including HFR1, LAF1, and HY5 (Seo et al., 2003; Jang et al., 2005; Yang et al., 2005). The stability of these positive factors is increased upon exposure to light, indicating a light-dependent inactivation of COP1 (Kami et al., 2010; Li et al., 2011). Here, we demonstrated that the phyA- and phyB-SPA interactions disturb the COP1/SPA complex under activating light conditions. Indeed, loss of SPA1 has been shown to enhance light responses, and it has been suggested that phyA might inactivate SPA1 in response to FR light (Hoecker et al., 1998). Moreover, multiple *spa*-null seedlings are hypersensitive to FR light, particularly in the absence of a functional SPA1, consistent with less activated phyA being required for inactivating the lower levels of SPA proteins in these lines (Balcerowicz et al., 2011). We propose that under light conditions where phyA and phyB accumulate in the nucleus, activated phyA and phyB bind to SPAs, disrupting the COP1-SPA interaction and thereby preventing the degradation of positive photomorphogenic factors (Figure 7). Even after disruption of the direct COP1-SPA interaction, COP1 can be retained in complex due to a direct interaction with phyA. Although the coiled coil domain of SPA1, which mediates the interaction with COP1 (Hoecker and Quail, 2001), is not blocked directly through SPA1-phyA binding, it is possible that phyA introduces a steric hindrance that interferes with binding of SPA1 to COP1.

Phytochromes binding to COP1/SPA may have additional effects, such as regulation of SPA protein turnover (Balcerowicz et al., 2011) or, hypothetically, modification of COP1 and SPAs, which may be important to ensure sustained downregulation of COP1/SPA activity in light-grown plants. It is interesting that the kinase-like domain of SPA1 was found to be essential for the interaction with phyA, as it has been shown that the coiled coil and WD-repeats produce a functional SPA1 but that the kinase-like domain is required for the destabilization of SPA1 in FR (Yang and Wang, 2006). SPA1 and SPA2 are destabilized in seedlings exposed to FR (Balcerowicz et al., 2011), and it is possible this is a consequence of the light-regulated interaction with phyA. Interestingly, phyA degradation in R is reduced in *spa123* triple mutant background (Debrieux et al., 2013), suggesting that binding of SPAs to activated phyA could contribute to light-enhanced phyA protein turnover, which may be important to avoid overactivation of the phyA downstream signaling

pathway in R. phyA is also stabilized in the *cop1-4* mutant in presence of sucrose, which is in contrast to *spa123*, where the effect occurs on standard Murashige and Skoog growth medium (Debrieux et al., 2013). Given that phyA degradation is only reduced but not inhibited in *spa123* and *cop1-4* and stabilization partially depends on sucrose, it seems unlikely that COP1/SPA are key components of the as yet unknown general phyA degradation mechanism.

A light-independent phyB-SPA1 interaction has been reported and proposed to enhance the activity of COP1/SPA1 in FR light, stabilizing HY5 and thereby counteracting phyA signaling (Zheng et al., 2013). Using altered conditions, including photoactive full-length phyB, we have shown the interaction between phyB and SPA1 to be dependent on light. Moreover, in yeast three-hybrid competition assays, light-activated phyB disrupted the direct interaction of COP1 and SPA1 that would presumably downregulate the activity of COP1/SPA1 in planta. Consistent with a negative effect of phyB on COP1/SPA1 activity in R, HY5 protein levels are increased under these conditions in a phyB-dependent manner (Osterlund et al., 2000). However, *HY5* transcript levels also are elevated upon exposure to light, making it difficult to establish to what extent stabilization of the protein and increased mRNA levels contribute to HY5 protein accumulation in R (Osterlund et al., 2000).

A direct COP1-phyA interaction has been reported (Seo et al., 2004; Viczián et al., 2012). However, these experiments are not consistent, showing either an interaction between the N-terminal PAS-GAF region of phyA and COP1 (Viczián et al., 2012) or between the PAS-A/PAS-B region of phyA and the COP1 WD-repeat domain (Seo et al., 2004). As neither interaction has been shown to be light dependent, they do not explain how phyA mediates a light-specific repression of COP1 activity, though there is potential for other regulation such as light-dependent phosphorylation to be involved. We observed that COP1 and phyA interact within NBs in planta; therefore, exclusion of COP1 from the complex is unlikely in FR light. Indeed, COP1 has been reported to reaccumulate in the nucleus in shade conditions that are rich in FR light (Pacín et al., 2013). It is fortunate that a COP1-phyA interaction cannot be detected in yeast using full-length phyA, as this allowed the detection of the light-induced phyA-mediated disruption of the COP1/SPA interaction. Interestingly, expression of the N terminus of phyA (1 to 406), which binds to COP1 independently of light, results in constitutive signaling in plants (Viczián et al., 2012). However, this fragment can bind both COP1 and SPA1 independent of light, making it difficult to establish which interaction of this fragment represses COP1 function in planta, i.e., the effect observed by Viczián et al. (2012) is not necessarily due to interaction of phyA 1-406 with COP1 but might be due to its binding to SPA proteins or both.

COP1, SPA1, and phyA are present in the same complex in NBs of FR-grown plants. Yet, in vivo experiments revealed that light-activated phyA binding to SPA1 reorganizes the complex, likely increasing the spatial distance and therefore disrupting the direct interaction between COP1 and SPA1. Given that phyA NB formation is observed only upon irradiation with light, even in lines expressing constitutively nuclear-localized phyA (Genoud et al., 2008), it seems likely that in planta binding of phyA to COP1 is Pfr dependent. Thus, binding of activated phyA to COP1 also may contribute to disruption of the direct interaction between COP1 and SPA1.

Both light-activated CRY1 and CRY2 have been shown to interact with SPA proteins, resulting in inactivation of the COP1/SPA complex and initiation of downstream signaling in response to blue light (Lian et al., 2011; Liu et al., 2011; Zuo et al., 2011). However, the mechanism of inactivation differs. CRY2 stabilizes the interaction of COP1 and SPAs, but nevertheless reduces the activity of the COP1/SPA complex (Zuo et al., 2011). By contrast, CRY1 inactivates the COP1/SPA complex by binding to SPA proteins and inhibiting their association with COP1 (Lian et al., 2011; Liu et al., 2011). Interestingly, it appears likely that phyA and CRY1 use a similar mechanism to downregulate the activity of the COP1/SPA complex; yet, phyA shares an interaction with the SPA1 kinase-like domain similar to CRY2 (Zuo et al., 2011), whereas CRY1 binds to the SPA1 WD-repeat domain (Liu et al., 2011). We have shown HFR1 protein accumulation in seedlings within 2 h of irradiation with FR, similar to previous studies where it was found that HFR1 is rapidly stabilized by blue light, peaking 2 h after light exposure (Duek et al., 2004). It seems likely that different photoreceptors employ a similar mechanism to trigger light-induced accumulation of HFR1, which is degraded in dark-grown seedlings in a COP1-dependent manner (Duek et al., 2004).

Here, we have shown that SPAs interact with phyA and phyB within NBs in planta, though we cannot exclude that they also interact in the nucleoplasm, which could not be determined by FRET-FLIM due to the lower abundance in this fraction. Many other components of light signaling have been found to form nuclear bodies as well; however, the function of these electron-dense structures is still unclear (Van Buskirk et al., 2012). Other photoreceptors, including the blue-light-absorbing cryptochromes, and the UV-B receptor UVR8, localize to nuclear bodies in light and either inactivate or alter the function of the COP1/SPA complex, potentially forming a converging point for light signaling pathways (Van Buskirk et al., 2012).

METHODS

Plant Material

Arabidopsis thaliana Columbia-0 (Col-0) *ProPHYA:PHYA-CFP* and *Pro35S:HA-YFP-SPA1* were created by *Agrobacterium tumefaciens*-mediated cotransformation of Col-0 with pPHYA40-PHYA and pPPO70v1HA-SPA1 (Davis et al., 2009); plasmids are described in the Supplemental Methods. *spa123 ProPHYA:PHYA-CFP* and *cop1-4 ProPHYA:PHYA-CFP* were obtained by transforming pPHYA40-PHYA into *spa1-7 spa2-1 spa3-1* (Balcerowicz et al., 2011) and *cop1-4* (McNellis et al., 1994) backgrounds, respectively. The lines *hfr1-4 Pro35S:LUC-HFR1*, *phyA-211 ProPHYA:PHYA-YFP*, *spa1-7 ProSPA1:HA-YFP-SPA1*, and *spa1-7 Pro35S:HA-YFP-SPA1* were created by Agrobacterium-mediated transformation of *hfr1-4* (Sessa et al., 2005), *phyA-211* (Reed et al., 1994), and *spa1-7* (Fittinghoff et al., 2006) with pCHF91-HFR1, pPPO30A-PHYA, pSPA1HAYFP-SPA1, and pPPO70v1HA-SPA1. The c-MYC-SPA1 line has been described previously (= *Pro35S:TAP-SPA1* = *Pro35S:2xIgG-BD-9xc-MYC-SPA1*; Saijo et al., 2003).

Growth Conditions

Wild tobacco (*Nicotiana benthamiana*) plants were greenhouse cultivated (temperature, 26°C day/19°C night; humidity, 62%; photoperiod, 14 h).

Germination of Arabidopsis seeds was induced by stratification on half-strength Murashige and Skoog agar plates for 4 d at 4°C, followed by 4 h white or R light induction. Subsequently, plates were transferred to either complete darkness, continuous FR (720 nm, 12 μmol m^{-2} s^{-1}, or 1 μmol m^{-2} s^{-1} where indicated for *spa1-7*), or continuous R light (670 nm, 30 μmol m^{-2} s^{-1}), each at 22°C.

Transient Transformation of *N. benthamiana* Leaves

The leaves of 4- to 6-week-old *N. benthamiana* plants were infiltrated with Agrobacterium C58 as previously described (Grefen et al., 2008). The p19 protein from tomato bushy stunt virus was used for suppression of transgene silencing (Voinnet et al., 2003). Sixteen hours after infiltration, *N. benthamiana* plants were transferred to complete darkness (26°C) for 2 d to allow the accumulation of phyA. Transient expression and localization of the fusion proteins in plant epidermal leaf cells were detected using epifluorescence microscopy or CCD camera visualization after spraying with 1 mM D-luciferin (*Pro35S:PHYA-LUC*). The constructs used for transient expression in *N. benthamiana* leaves are described in the Supplemental Methods; expression of constructs in *N. benthamiana* was driven by the 35S promoter.

FRET-FLIM Analysis

All FLIM measurements were performed as previously described (Wanke et al., 2011), with the following modifications for CFP-YFP FRET. A pulsed 440-nm diode laser (Picoquant LDH-D-C-440), operating at a repetition rate of 20 MHz, was used for excitation, in conjunction with LD01-439/8-12.5 (Semrock) cleanup interference filters. A dichroic beam splitter plate (λ_{cut-on} = 455 nm at 45° incident angle) was used with a long-pass interference filter (LP02-458RU-25) and a band-pass interference filter (BrightLine Basic FF01-469/35-25; Semrock) to exclusively detect the donor (CFP) signal. To survey the cell nuclei, FLIM images were obtained by raster scanning the samples using a feedback controlled piezo-driven sample stage (P-517.3CD; PI Physik Instrumente). Only photons originating from nuclear bodies were selected for analysis. Time-correlated single-photon-counting histograms were deconvolved from the instrument response function and fitted to exponential decays to provide the average lifetime.

Yeast Interaction Assays

All yeast two- and three-hybrid plasmids (described in the Supplemental Methods) were cotransformed into *Saccharomyces cerevisiae* strain Y190 (Harper et al., 1993) using a Frozen-EZ yeast transformation kit (ZymoResearch), followed by growth selection on synthetic media lacking leucine and tryptophan. Transformed yeast were suspended in sterile double-distilled water and, under green light (525 nm) conditions, 5 μL plated onto selective media lacking leucine and tryptophan (and methionine for yeast three-hybrid assays) supplemented with 20 μM phycocyanobilin (purified from Spirulina as previously described; Kunkel et al., 1993). Plates were incubated for 48 h (yeast two-hybrid assay) or 72 h (yeast three-hybrid assay) at 26°C in either darkness, constant R light (670 nm, 1.7 μmol m^{-2} s^{-1}), or FR light (720 nm, 13 μmol m 2 s^{-1}). X-Gal filter lift assays were performed as previously described (Breeden and Nasmyth, 1985) except yeast were lifted from plates and freeze/thawed five times with liquid N_2 under green light. For quantitative *ortho*-nitrophenyl-β-galactoside (ONPG) assays, yeast was grown as above, harvested in green light, and used for liquid ONPG assays as previously described in the Clontech yeast two-hybrid manual.

Immunolocalization

Arabidopsis seedlings were fixed in microtubule stabilizing buffer with a formaldehyde concentration of 4% (w/v) for 45 min, followed by 8% for a further 120 min. Hypocotyls were embedded in 10% (w/v) gelatin and then infiltrated with a solution of 2.1 M sucrose and 1.8% (w/v) polyvinylpyrrolidone and mounted on stubs. Samples were frozen in liquid nitrogen, and ultrathin (70 nm) sections were cut at −115°C using a cryo-ultramicrotome (Leica). Sections were transferred to Pioloform and carbon-coated grids and blocked for 30 min in PBS, 0.5% (w/v) BSA, and 0.5% (w/v) milk powder. Grids were probed with rabbit α-phyA serum (Agrisera; 1:300 in blocking buffer) for 60 min and washed six times with blocking buffer. Bound antibodies were detected with 6-nm gold particle labeled protein-A (1:50 in blocking buffer; Aurion). Grids were stained with 1% (v/v) uranyl acetate and embedded in a thin layer of methyl cellulose containing 0.3% (v/v) uranyl acetate and imaged with a transmission electron microscope (Leo 906).

Luciferase Quantification

Four-day-old seedlings were harvested and frozen in liquid N_2 under green light. Tissue was disrupted using glass beads and a Silamat while frozen and proteins extracted in LUCI buffer (100 mM K_2PO_4, pH 7.8, 0.05% [v/v] Tween 20, protease inhibitor cocktail [Sigma-Aldrich P2714], 20 μM MG132, and 1 mM DTT) under green light. Lysate (100 μL) was then assayed in triplicate in a luminometer, injecting 50 μL LUCII buffer (80 mM glycyl-glycine, 60 mM ATP, and 40 mM $MgSO_4$, pH 7.8) and 100 μL 10 mM D-luciferin. Protein concentration was determined by amido-black as previously described (Popov et al., 1975).

Luciferase Assay

Yeast cultured on selective media supplemented with 20 μM PCB and grown for 2 d in constant R light (670 nm, 1.7 μmol m^{-2} s^{-1}) were sprayed with 5 mM D-luciferin and visualized by 5-min exposure with a CCD camera.

Immunoblotting

Seedlings were treated as for luciferase quantification, except proteins were extracted in 100 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, 10 mM NaF, 15 mM glycero-phosphate, 50 μM MG132, protease inhibitor cocktail (Sigma-Aldrich P2714), 0.1% SDS, and 1 mM DTT. Protein concentration was determined as above, and 5 μg each sample separated by 8% SDS-PAGE electrophoresis, blotted to polyvinylidene fluoride membrane, blocked, and probed. Primary antibody (LUC-1 Sigma-Aldrich L2164) was used at 1:2000 overnight at 4°C, secondary antibody (α-mouse AP Vector Laboratories) was used at 1:10,000 for 1 h, and detection performed with 5-bromo-4-chloro-3-indolyl phosphate and Nitro blue tetrazolium.

In Vivo Coimmunoprecipitation Assays

For the in vivo coimmunoprecipitation assay, 4-d-old dark-grown seedlings expressing SPA1 tagged with an alternative TAP tag containing 9xc-MYC (Saijo et al., 2003) were pretreated with 50 μM MG132 for 5 h and either kept in darkness or given a pulse of 3000 μmol m^{-2} R light followed by 10-min dark incubation. Total proteins were extracted with 0.8 mL native extraction buffer (100 mM phosphate buffer, pH 7.8, 150 mM NaCl, 0.1% Nonidet P-40, 1× protease inhibitor [Sigma-Aldrich P9599], 1 mM PMSF, 50 μM MG132, 5 μM β-mercaptoethanol, 25 mM β-glycerophosphate, 10 mM sodium fluoride, and 2 mM sodium orthovanadate) and cleared by centrifugation at 16,000*g* for 15 min at 4°C. For each sample, 20 μL Dynabeads (Life Technologies 10002D) were preincubated with 1 μg α-MYC antibody (Cell Signaling Technology; 2276S) at 4°C for an hour and washed twice with the native extraction buffer. Total protein extracts (500 μg) along with antibody bound beads in 1 mL total volume were incubated at 4°C in the dark for 1 h. The beads were washed three

times with the binding buffer (with 0.2% Nonidet P-40), dissolved in 1× SDS loading buffer, and incubated at 65°C for 10 min. The immunoprecipitated proteins were separated on 6.5% SDS-PAGE gel and transferred on to polyvinylidene fluoride membrane. α-phyA (073D) (1:500) and α-MYC (1:5000) (Sigma-Aldrich SAB4700447) antibodies were used to detect phyA and TAP-SPA1 proteins.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: At2g32950 (COP1), At2g37678 (FHY1), At1g02340 (HFR1), At1g09570 (PHYA), At2g18790 (PHYB), At2g46340 (SPA1), At4g11110 (SPA2), At3g15354 (SPA3), and At1g53090 (SPA4).

Supplemental Data

Supplemental Figure 1. phyA Colocalizes with SPA3 and SPA4 in *Nicotiana benthamiana* Nuclear Bodies.

Supplemental Figure 2. Light-Activated phyA Interacts with SPA1 and SPA2 in Yeast.

Supplemental Figure 3. Pro35S:HA-YFP-SPA1 Rescues spa1-7.

Supplemental Figure 4. Immunohistochemistry of phyA and phyA-YFP Nuclear Bodies.

Supplemental Figure 5. Truncations and Mutants of phyA and SPA1 Are Stable in Yeast.

Supplemental Figure 6. Immunoblot Detection of Yeast Three-Hybrid Proteins from Figure 4.

Supplemental Figure 7. Reorganization of the COP1/SPA1 Complex by phyA upon Irradiation with Light.

Supplemental Figure 8. HFR1 Accumulates in FR Light.

Supplemental Methods. Yeast Interaction Assays, Immunoblotting, Immunohistochemistry, and Cloning of Constructs.

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AUTHOR CONTRIBUTIONS

D.J.S., C.M., S.z.O.-K., F.S., Y.-D.S., L.Z., E.H., and A.H. designed research. D.J.S., C.M., S.z.O.-K., B.E., L.Z., P.J., and Y.-D.S. performed research and analyzed data. D.J.S., C.M., S.z.O.-K., Y.-D.S., L.Z., E.H., and A.H. wrote the article.

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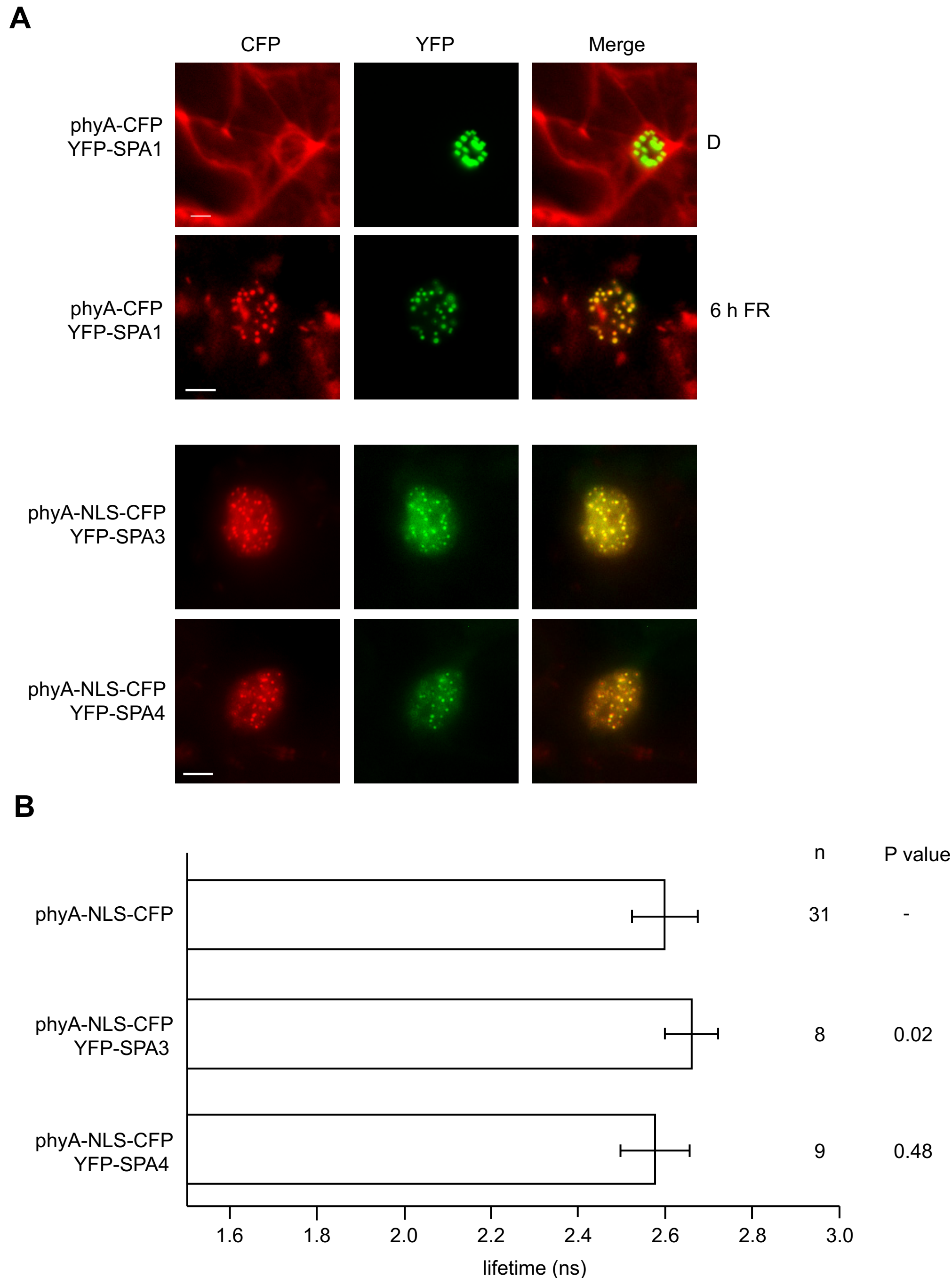
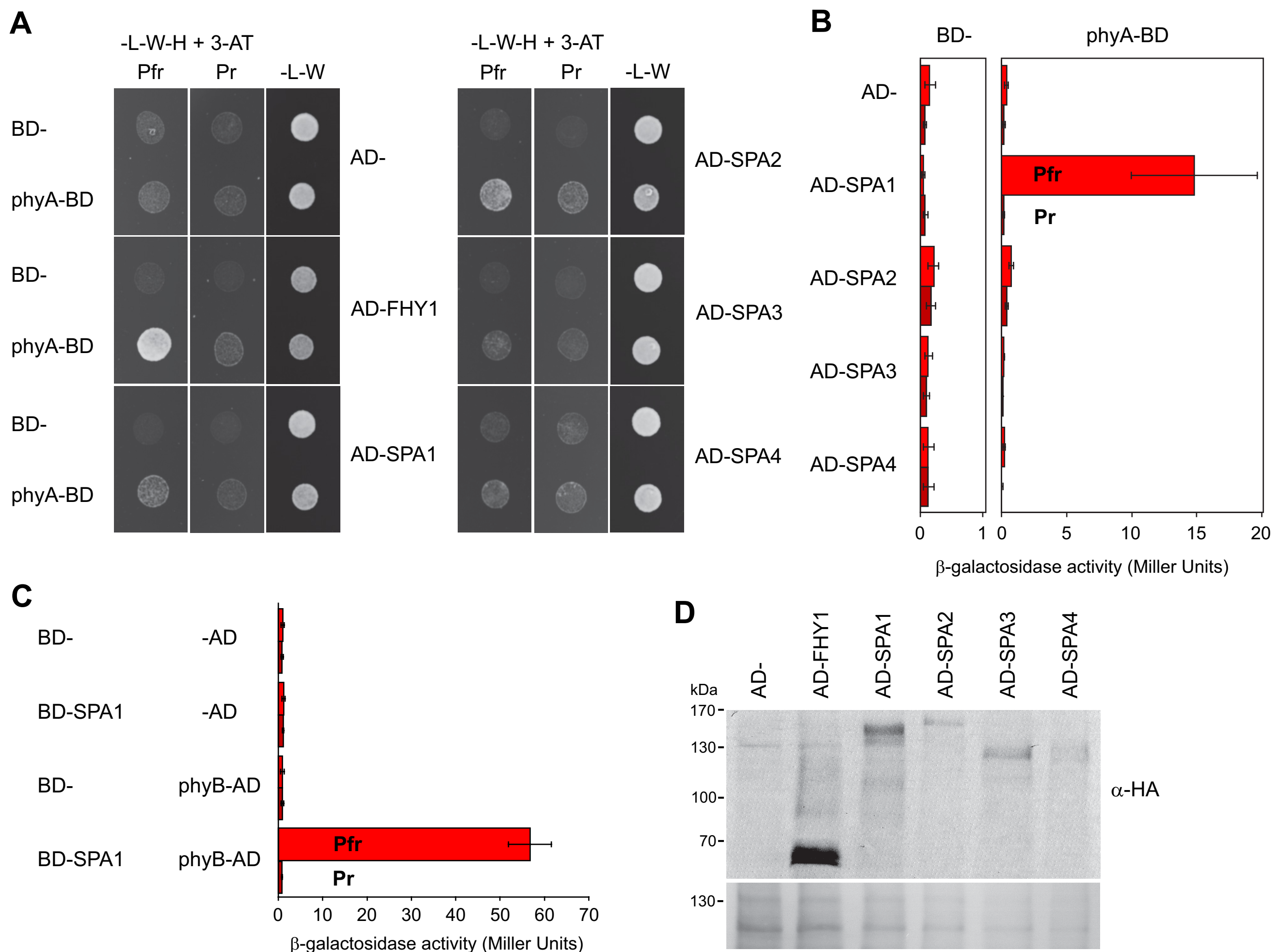
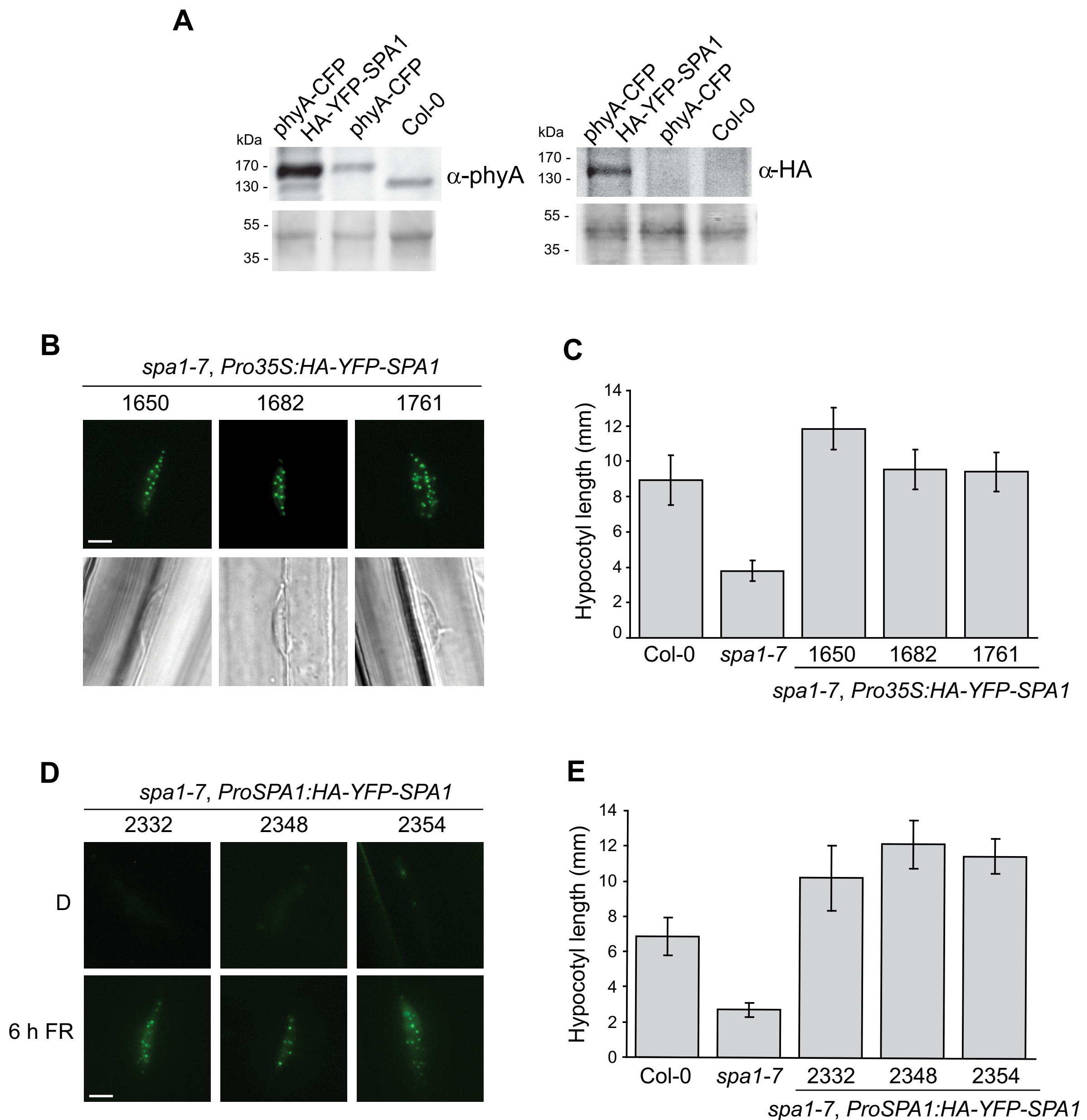


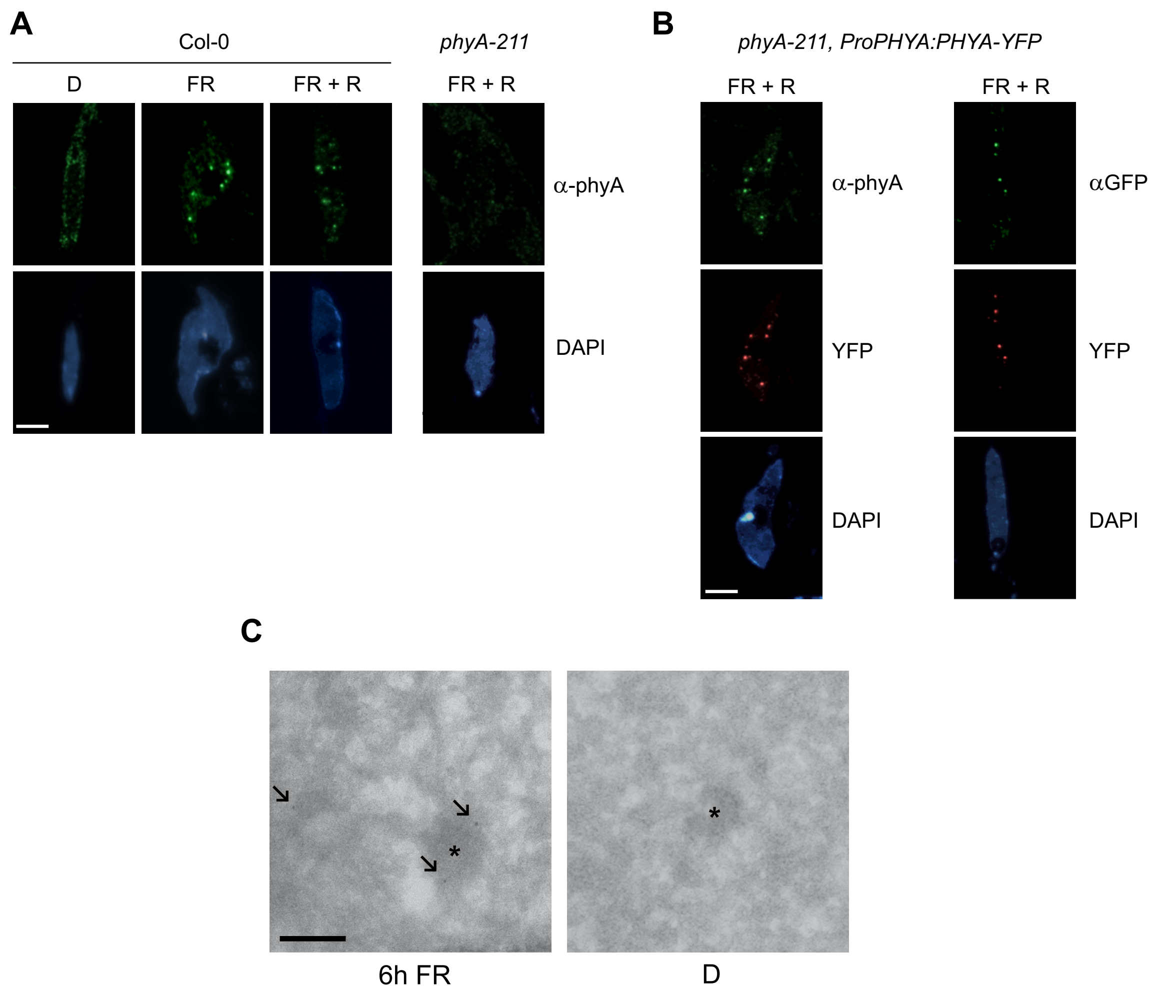
Supplemental Figure 1. PhyA co-localizes with SPA3 and SPA4 in *Nicotiana benthamiana* nuclear bodies. **(A)** Epi-fluorescence microscopy detection of phyA-CFP or phyA-NLS-CFP transiently co-expressed with YFP-SPA1, -SPA3, or -SPA4 in *Nicotiana benthamiana* leaf epidermal cells. Plants expressing phyA-CFP were exposed to FR light (18 µmolm^{-2}s^{-1}) for 6 h prior to imaging, whereas those transformed with phyA-NLS-CFP were kept under constant darkness (D) prior to microscopy. The scale bars indicate 10 µm. **(B)** FRET-FLIM analysis of nuclear body localized phyA and SPA3/SPA4 CFP and YFP fusions transiently expressed in *Nicotiana benthamiana*. The fluorescence lifetime of the donor (CFP) is shown. n = number of measurements. Error bars show one standard deviation. P values indicate t-test analysis for statistically significant differences. Fusion proteins used in A and B were expressed under the control of the 35S promoter.



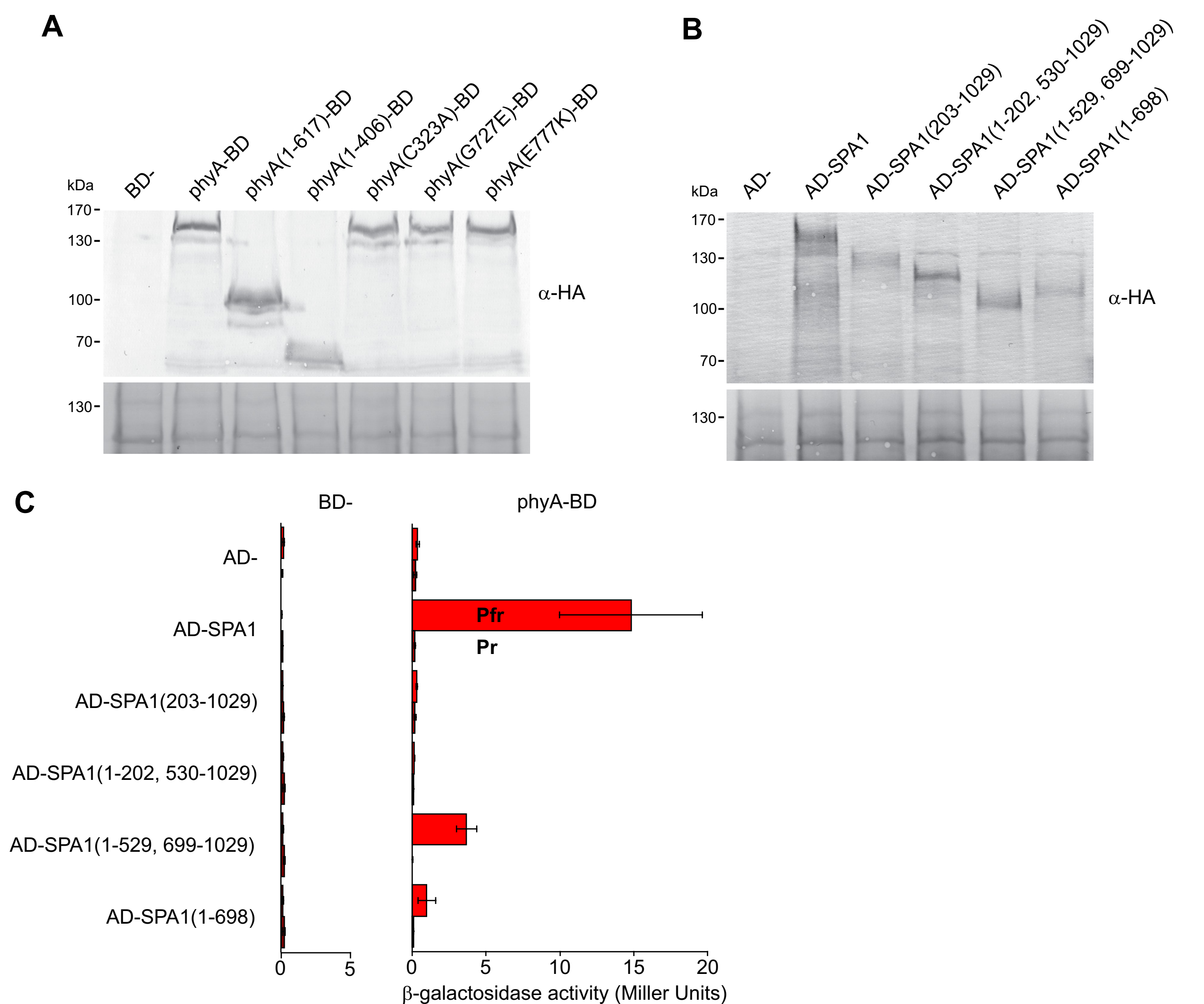
Supplemental Figure 2. Light-activated phyA interacts with SPA1 and SPA2 in yeast. **(A)** Yeast two-hybrid protein-protein interaction assay. The phyA-GAL4-DNA binding domain (phyA-BD) fusion was co-expressed with GAL4-activation domain (AD-) fusions of FHY1 and SPA1-4. Yeast were grown on selective media lacking histidine, supplemented with the histidine biosynthesis inhibitor 3-amino triazole (3-AT) and phycocyanobilin (PCB), under constant R (Pfr) or FR (Pr) light to assay activation of the HIS-reporter gene. **(B)** Yeast two-hybrid protein-protein interaction of phyA-BD and AD-SPA1-4. Yeast were grown on chromophore-supplemented plates for 48 h under either constant R (bright-red, Pfr) or FR (dark-red, Pr) light. Interaction was detected by a liquid o-nitrophenyl-β-galactoside (ONPG) assay. Values are the average of nine assays; error bars display one standard deviation. **(C)** as for (B) using BD-SPA1 and phyB-AD. **(D)** Immunoblot detection of yeast expressed FHY1 and SPA1-4. Yeast were harvested from chromophore supplemented plates that had been incubated for 48 h under constant R light. FHY1 and SPA1-4 AD-fusions contain a HA tag, and an α-HA antibody was used to detect these AD fusions in yeast protein extracts. The lower pane shows the membrane stained with amido-black as a loading control.



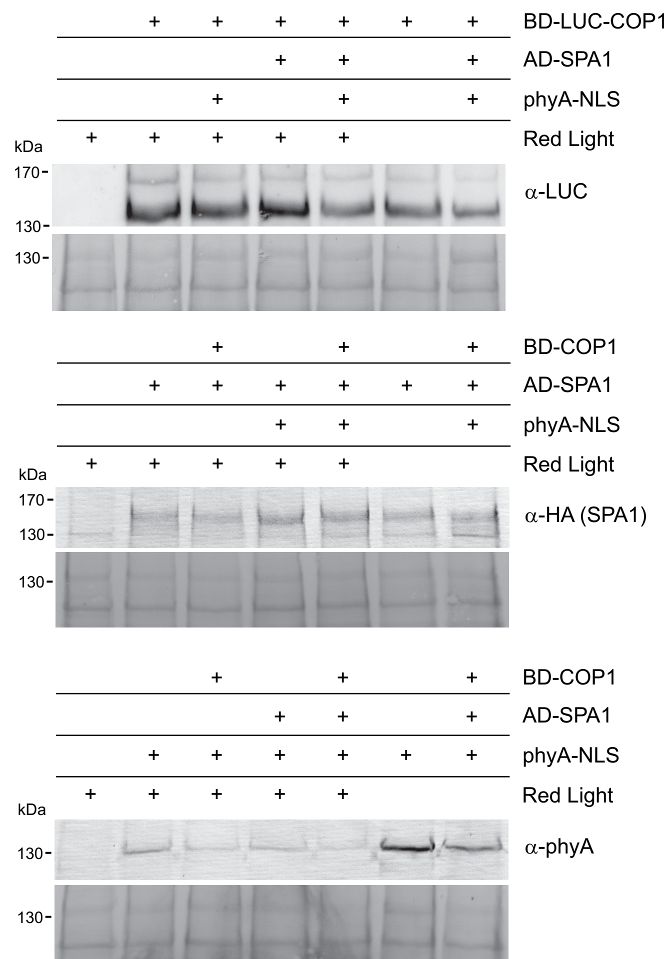
Supplemental Figure 3. *Pro35S:HA-YFP-SPA1* rescues *spa1-7*. **(A)** Immunoblot detection of phyA-CFP and HA-YFP-SPA1 proteins expressed in stable transformed Arabidopsis. The lower pane shows the membrane stained with amido-black as a loading control. **(B)** Localization of HA-YFP-SPA1 in dark-grown *spa1-7 Pro35S:HA-YFP-SPA1*. **(C)** Hypocotyl measurements of 4-day-old Arabidopsis *spa1-7 Pro35S:HA-YFP-SPA1* seedlings grown in weak FR (1 μmolm^{-2}s^{-1}). **(D)** Localization of HA-YFP-SPA1 expressed from the native *SPA1* promoter in *spa1-7 ProSPA1:HA-YFP-SPA1* seedlings grown in darkness, or darkness followed by 6 h FR (10 μmolm^{-2}s^{-1}). **(E)** Hypocotyl measurements of 4-day-old Arabidopsis *spa1-7 ProSPA1:HA-YFP-SPA1* seedlings grown in weak FR (1 μmolm^{-2}s^{-1}). White scale bars indicate 4 μm. Error bars display one standard deviation for measurements from 25 or more seedlings. Data for three independent transgenic lines are shown in (B-E).



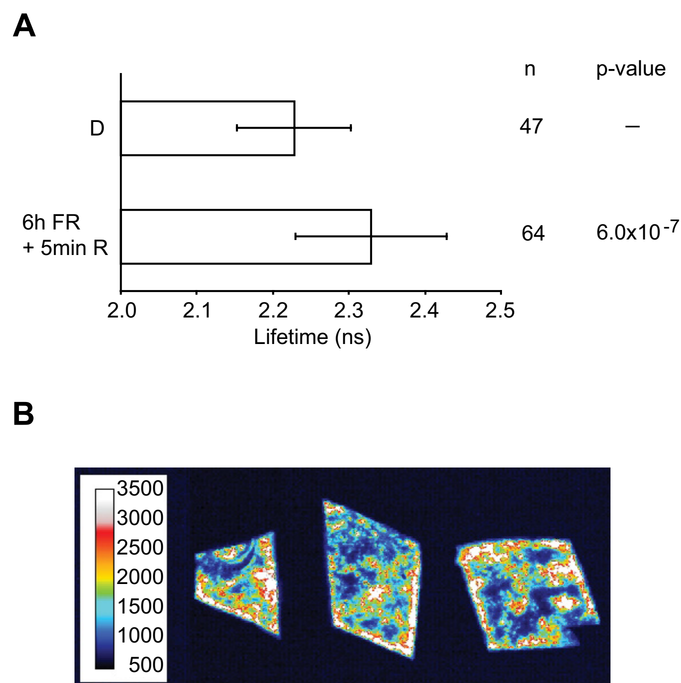
Supplemental Figure 4. Immuno-histochemistry of phyA and phyA-YFP nuclear bodies. **(A)** Detection of endogenous phyA in hypocotyl cell nuclei. Seedlings were grown in darkness for 4 d, followed by either no light (D), 6 h FR light (18 μmolm^{-2}s^{-1}) (FR), or 6 h FR followed by 10 min R light (22 μmolm^{-2}s^{-1}) (FR + R) and subsequently fixed with formaldehyde. phyA was detected using α-phyA and a Cy3-coupled secondary antibody. **(B)** Detection of phyA-YFP. As for (A), except YFP was additionally detected with α-GFP and the YFP fluorescence visualized directly. DAPI staining of DNA used to show nuclei. (A) and (B) The scale bar indicates 4 μm. **(C)** Immuno-electron microscopy-localization of phyA in wild-type Arabidopsis Col-0 hypocotyl nuclei. Seedlings were grown in darkness for 4 d and treated with either 6 h FR light followed by 5 min R light, or constant darkness prior to fixation. Endogenous phyA was probed with α-phyA antibodies, and detected with protein A-labelled 6-nm gold particles (indicated by arrows). Nuclear bodies are indicated with *. Black scale bar indicates 200 nm.



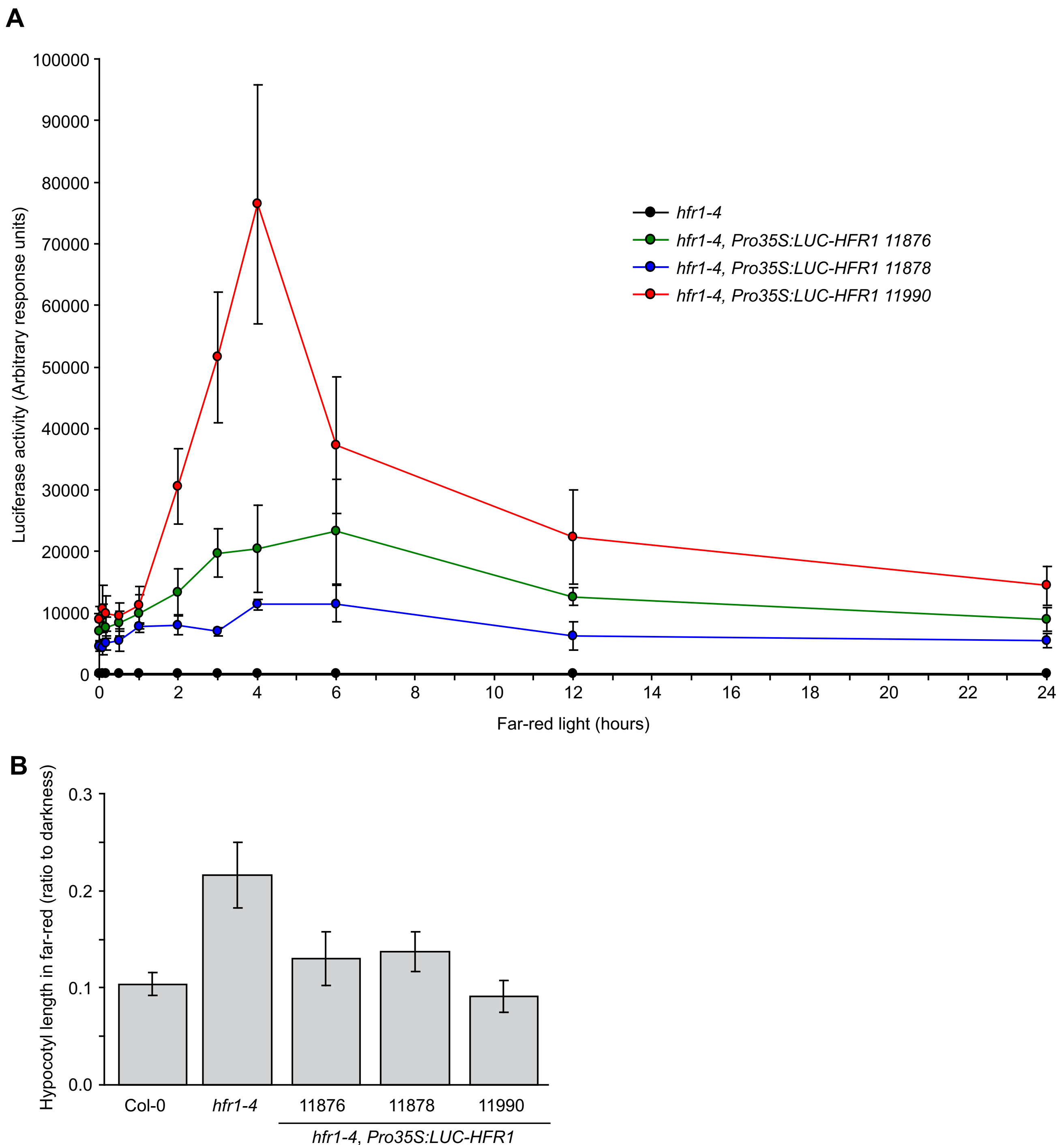
Supplemental Figure 5. Truncations and mutants of phyA and SPA1 are stable in yeast. **(A, B)** Yeast cells were harvested from chromophore-supplemented plates that had been incubated for 48 h under constant R light. All AD-SPA1 and phyA-BD fusions contain a HA tag, and an α-HA antibody was used to detect the SPA1 and phyA fusions in yeast protein extracts. The lower panels show the membrane stained with amido-black as a loading control. **(A)** Immunoblot detection of yeast-expressed phyA truncations and single amino acid substitution mutants. **(B)** Immunoblot detection of yeast-expressed SPA1-truncated proteins. **(C)** Yeast two-hybrid protein-protein interaction of phyA-BD and AD-SPA1 truncations. Yeast were grown under either constant R (bright-red, Pfr) or FR (dark-red, Pr) light. Interaction was detected by a liquid o-nitrophenyl-β-galactoside (ONPG) assay. Values are the average of nine assays; error bars display one standard deviation.



Supplemental Figure 6. Immunoblot detection of yeast three-hybrid proteins from Figure 4. Yeast cells were harvested from chromophore supplemented plates that had been incubated for 72 h under either constant R light or darkness. The AD-SPA1 fusion contains an HA; α-HA, α-LUC, and α-phyA antibodies were used to detect AD-SPA1, BD-LUC-COP1, and phyA-NLS in yeast protein extracts. Yeast co-expressed combinations of either BD-/BD-COP1/BD-LUC-COP1 with AD-/AD-SPA1 and -/phyA-NLS. Lower panels show amido-black stained membranes as a loading control. The upper band detected by α-LUC is the expected molecular weight for the full-length COP1-fusion protein (155 kDa).



Supplemental Figure 7 - Reorganization of the COP1/SPA1 complex by phyA upon irradiation with light. **(A)** FRET-FLIM analysis of the disruption of the interaction between COP1 and SPA1. CFP-SPA1, YFP-COP1 and phyA-LUC were co-transformed into *Nicotiana bethamiana* and plants were grown in darkness (D) or darkness followed by 6 h FR and 5 min R pulse to activate phyA nuclear transport and NB formation. Prior to microscopy, leaves were fixed to prevent effects of irradiance with fluorescent light during FRET measurement on the COP1/SPA1 complex. The fluorescence lifetime of the donor (CFP) is shown. Error bars show one standard deviation. n = number of measurements. P values indicate t-test analysis for statistically significant differences. **(B)** Detection of phyA-LUC in co-transformed *Nicotiana bethamiana*. To confirm expression of phyA-LUC, leaves were sprayed with 1 mM D-luciferin and detected by 5 min exposure using a CCD camera. Color scale indicates relative light emittance. Expression of constructs in A and B was driven by the 35S promoter.



Supplemental Figure 8. HFR1 accumulates in FR light. **(A)** Quantification of LUC-HFR1 abundance in three independent transgenic Arabidopsis *hfr1-4 Pro35S:LUC-HFR1* lines. Seedlings were grown in darkness after 6 h germination induction with white light, and transferred to FR light (10 $\mu molm^{-2}s^{-1}$) at various time points prior to harvesting at 4 d (96 h) post germination induction. Error bars show one standard deviation of nine measurements from three biological replicates. **(B)** Hypocotyl lengths of 4-day-old Arabidopsis *hfr1-4 Pro35S:LUC-HFR1* seedlings grown in continuous FR light (10 $\mu molm^{-2}s^{-1}$) following 16 h darkness, as a ratio to hypocotyl length in darkness. Error bars display one standard deviation of 30 or more seedlings.

SUPPLEMENTAL METHODS

Yeast interaction assays

For histidine-reporter assays, yeast harvested from overnight cultures were washed and suspended in sterile ddH_2O to an OD_{600} of 0.1. 3 µL spots were plated onto synthetic media lacking leucine, tryptophan and histidine, supplemented with 20 µM phycocyanobilin (PCB) and 20 mM 3-amino triazole, or control plates lacking leucine and tryptophan. Plates were incubated for four days at 26 °C in either constant R light (670 nm, 1.7 µmolm^{-2}s^{-1}), or FR light (720 nm, 13 µmolm^{-2}s^{-1}). Control plates were incubated for 48 h in normal light conditions. For o-nitrophenyl-β-galactoside (ONPG) assays yeast were cultured on plates lacking leucine and tryptophan, supplemented with 20 µM phycocyanobilin. Plates were incubated under constant R or FR light as above for 48 h at 26 °C. ONPG assays were otherwise performed as described in the Clontech yeast two-hybrid manual.

Immunoblotting

Total protein was extracted from yeast as previously described (Printen and Sprague, 1994), except yeast were harvested from plates supplemented with 20 µM phycocyanobilin, grown for 48 h at 26 °C in either darkness or constant R light (670 nm, 1.7 µmolm^{-2}s^{-1}). Total protein was extracted from four day old Arabidopsis seedlings as previously described (Kircher et al., 2002). Protein transferred to membranes was detected by amido-black staining. Blocked membranes were incubated with primary antibodies for 16 h at 4 °C. Primary antibodies were diluted in 50 mM Tris:HCl pH 7.5, 150 mM NaCl, 0.005 % (v/v) Tween-20, 5 % (w/v) milk powder, with the following exceptions: 500 mM NaCl and 0.05 % Tween-20 were used for blots of Arabidopsis extracts, and 2 % (w/v) ECL advance blocking agent used in place of milk powder for detection of BD-LUC-COP1. Antibody dilutions were as follows: α-HA (Covance or Roche) 1:1000, α-phyA (Agrisera, for yeast) 1:2000, α-LUC (Sigma) 1:2000, and α-phyA ((Hiltbrunner et al., 2006), for plants) 1:3000. Secondary antibodies were used at either 1:50000 (alkaline-phosphatase conjugate, Sigma), 1:50000 (Horseradish-peroxidase conjugate, GE Healthcare) for yeast, or 1:1500 (Horseradish-peroxidase conjugate, Santa Cruz Biotechnology) for detection of Arabidopsis proteins. Immunoblots were developed using either Nitrotetrazolium Blue and Bromo-chloro-indolyl phosphate, ECL advance

reagents (detection of BD-LUC-COP1, GE Healthcare), or ECL reagents (Pierce).

Immuno-histochemistry

Sections were prepared as for immunogold-labeling, except 400-nm sections were cut at - 80 °C and transferred to coverslips. Sections were probed with either rabbit α-phyA (1:300, Agrisera) or rabbit α-GFP (1:500, Abcam) and washed 6 times. Bound antibodies were detected with goat α-rabbit IgG-Cy3 conjugate (1:400, Dianova). Following washes with phosphate buffered saline, sections were stained with DAPI (1 μg/mL, 4',6-Diamidino-2-phenylindole dihydrochloride, Sigma) for 10 min to detect DNA, embedded in Mowiol (Sigma), and visualized with an epi-fluorescent microscope (Zeiss). The primary antibodies were omitted in control experiments, resulting in negligible background.

Cloning of constructs

The plant expression vectors pCHF40-PHYA (encoding *Pro35S:PHYA-CFP-TerRbcS*) and pPHYA40-PHYA (encoding *ProPHYA:PHYA-CFP-TerRbcS*) were previously described (Genoud et al., 2008).

pCHF40-PHYA-NLS (encoding *Pro35:PHYA-NLS-CFP-TerRbcS*) was created by amplifying the C-terminal region of PHYA-NLS present in pPHYA-PHYA-NLS-GFP5 (Genoud et al., 2008) by PCR using 5'-TTA CAC CAT CCG GAG GTC AG-3' and 5'-GGA CTA GTT GCG GCC GCT CCT CCA ACC T-3', cut with XbaI/SpeI, and used to replace the XbaI/SpeI fragment of PHYA in the intermediary vector pBS II KS-PHYA (Hiltbrunner et al., 2005). PHYA-NLS was subsequently cut with BamHI/SpeI and cloned into BamHI/XbaI cut pCHF40 (Hiltbrunner et al., 2005).

To generate an eYFP tagged form of PHYA-NLS, the fragment coding for PHYA-NLS was cut from pBS II KS-PHYA-NLS using BamHI/SpeI and cloned into the BamHI/XbaI sites of pPPO30 (Rausenberger et al., 2011).

The plant expression vector pPPO70v1HA (encoding *Pro35S:HA-YFP-BamHI-AvrII-XbaI-TerRbcS*) was generated by first cutting pCHF5 (Hiltbrunner et al., 2005) with PmeI/NcoI, and ligating in a StuI/NcoI fragment from pYES2 (Invitrogen) to generate pCHF5v1. pWCO35 (Rausenberger et al., 2011) was then cut with PvuII/PstI and this fragment ligated into PmlI/SbfI cut pCHF5v1 to generate pPPO5v1. Finally, eYFP was amplified by PCR from pPPO30 using 5'-GAA GAT CTA AAA ATG GCC TAC CCA TAC GAC GTA CCA GAT TAC GCT GCT AGC ATG GTG AGC AAG GGC GAG-3'/5'-GGA CTA

GTT ATC TAG AGC CCT AGG ATC CGC CTT GTA CAG CTC GTC CAT G-3', cut with BglII/SpeI and cloned into BamHI/XbaI cut pPPO5v1 to generate pPPO70v1HA.

SPA1 was amplified by PCR with 5'-GCT CTA GAA AAA TGC CTG TTA TGG AAA GAG-3' and 5'-GCT CTA GAA ACA AGT TTT AGT AGC TTC-3' from cDNA clone pda17902 (Riken), cloned into pBS II KS (pBS II KS-SPA1), cut with XbaI and cloned into the AvrII/XbaI sites of pPPO70v1HA to generate pPPO70v1HA-SPA1 (*Pro35S:HA-YFP-SPA1-TerRbcS*), or into the SpeI site of pCHF40 to generate pCHF40-SPA1 (*Pro35S:CFP-SPA1-TerRbcS*).

SPA2/3/4 were each amplified by PCR with 5'-ACG CGG ATC CAA AAA TGA TGG ATG AGG GAT CAG T-3'/5'-ACG CAC TAG TGA CCA ACT GTA GAA CTT TGA TT-3' (SPA2), 5'-ACG CGG ATC CAA AAA TGG AAG GTT CTT CAA ATT CTA ACT-3'/5'-ACG CAC TAG TAG TCA TCA TCT CCA GAA TTT TTA TG-3' (SPA3), and 5'-ACG CGG ATC CAA AAA TGA AGG GTT CTT CAG AAT CTA-3'/5'-ACG CAC TAG TTA CCA TCT CCA AAA TCT TGA TAT TG-3' (SPA4) from cDNA clones obtained from Ute Hoecker (University of Cologne, Germany), cut with BamHI/SpeI and cloned into the BamHI/XbaI sites of pPPO70v1HA.

The FRET positive control pCHF30-CFP (*Pro35S:CFP-YFP-TerRbcS*), was generated by ligation of BamHI/SpeI cut CFP from pCHF40 (Hiltbrunner et al., 2005) into BamHI/XbaI cut pCHF30 (Hiltbrunner et al., 2006).

pSPA1-HA-YFP-SPA1 is a T-DNA vector containing a *ProSPA1:HA-YFP-SPA1-TerRbcS* cassette and was obtained as follows. A 1672-bp SPA1 promoter fragment including the first 12 bp of the SPA1 coding sequence was PCR amplified from genomic Col-0 DNA using the primers 5'-CAT GCC ATG GGA TAC AAT TAT TGG GAG CTA TTA GTC-3' and 5'-CGG GAT CCT CCA TAA CAG GCA TCA ACA CTC-3'. This fragment was cut with NcoI/BamHI and ligated into the NcoI/BamHI site of pCHF5 (Hiltbrunner et al., 2005) resulting in pSPA1-1672. In parallel, HA-YFP was amplified by PCR from pPPO70v1HA-SPA1 using primers including BglII (5'-CAT GCC ATG GCA TGG AAG ATC TTA TGG CCT ACC CAT ACG ACG-3') and BamHI/AvrII/SpeI (5'-GAC TAG TTA CCT AGG TGC CGG ATC CGC CTT GTA CAG CTC GTC CAT GC-3') sites, respectively. The PCR fragment was then cut with BglII/SpeI and ligated into the BamHI/XbaI site of pSPA1-1672 to obtain pSPA1-1672-HA-YFP. Next, SPA1 was cut from pBS II KS-SPA1 using XbaI and ligated in sense orientation into the AvrII site of pSPA1-1672-YFP, resulting in pSPA1-1672-HA-YFP-SPA1. Finally, a 2260 bp SPA1 promoter fragment was PCR amplified from

genomic Col-0 DNA using 5'-CAT GCC ATG GTT TAA ACC TAG GGA GCA GAG AAA ATA ATA CAA CAT GTT GCT G-3' and 5'-CGG GAT CCT CCA TAA CAG GCA TCA ACA CTC-3'. This fragment was cut with PmeI/AatII and ligated into the PmeI/AatII site of pSPA1-1672-HA-YFP-SPA1 to obtain pSPA1-HA-YFP-SPA1.

pPPO70v1HA-COP1 is a T-DNA vector containing a *Pro35S:HA-YFP-COP1-TerRbcS* cassette. COP1 was PCR amplified from total Col-0 cDNA with the primers 5'-GAA GAT CTA AAA ATG GAA GAG ATT TCG ACG-3' and 5'-GGA CTA GTC GCA GCG AGT ACC AGA ACT TTG-3'. The PCR fragment was then cut with BglII/SpeI and ligated into the BamHI/XbaI site of pPPO70v1HA.

pCHF91-HFR1 is a T-DNA vector containing a *Pro35S:LUC+-HFR1-TerRbcS* cassette. Firefly luciferase (LUC+) was PCR amplified from *ProPIF3:LUC+* (Viczian et al., 2005) using the primers 5'-AAG ATC TAA AAA TGG AAG ACG CCA AAA ACA-3' and 5'-GGA CTA GTT ATC TAG AGC TTA CCT AGG ATC CGC CAC GGC GAT CTT TCC GCC C-3'. The PCR fragment was cut with BglII/SpeI and ligated into the BamHI/XbaI site of pCHF5 (Hiltbrunner et al., 2005), resulting in pCHF91 (*Pro35S:LUC+-BamHI-AvrII-XbaI-TerRbcS*). The primers 5'-CGC GGA TCC AAA AAT GTC GAA TAA TCA AGC TTT-3' and 5'-GGA CTA GTT AGT CTT CTC ATC GCA TGG G-3' where then used to amplify the HFR1 coding sequence from total Arabidopsis cDNA. The PCR fragment was cut with BamH/SpeI and ligated into the BamHI/SpeI site of pBluescript II KS (Stratagene), from which it was cut with BamHI/SpeI and ligated into the BamHI/XbaI site of pCHF91.

pCHF90-PHYA is a T-DNA vector containing a *Pro35S:PHYA-LUC+-TerRbcS* cassette. LUC+ was amplified by PCR from *ProPIF3:LUC+* using the primers 5'-CGC GGA TCC CGG CTC TAG AAT GGA AGA CGC CAA AAA CA-3' and 5'-GGA CTA GTT ACA CGG CGA TCT TTC CGC CC-3'. The PCR fragment was cut with BamHI/SpeI and ligated into the BamHI/XbaI site of pCHF5, resulting in pCHF90 (*Pro35S:BamHI-XbaI-LUC+-TerRbcS*). PHYA was then cut from pBS II KS-PHYA (Hiltbrunner et al., 2005) using BamHI/SpeI and ligated into the BamHI/XbaI site of pCHF90, resulting in pCHF90-PHYA.

pCHF40-PHYB is a T-DNA vector containing a *Pro35S:PHYB-eCFP-TerRbcS* cassette. The PHYB coding sequence was amplified from *Pro35S:PHYB-GFP* (Hiltbrunner et al., 2005) using the primers 5'-CCC AAG CTT CTA GAA AAA TGG TTT CCG GAG TCG GG-3' and 5'-GGG GTA CCT TAT CTA GAA TAT GGC ATC ATC AGC ATC A-3'. The PCR fragment was then digested with XbaI and ligated in sense orientation into the XbaI site of pCHF40 (Hiltbrunner et al., 2005).

pPPO30A-PHYA is a T-DNA vector containing a *ProPHYA:PHYA-eYFP-TerRbcS* cassette and has been described previously (Rausenberger et al., 2011).

All pCHF T-DNA vectors confer resistance to Basta; pPPO T-DNA vectors contain a mutated version of *PPO* as selection marker that results in resistance to Butafenacil/Inspire. Selection of transgenic plants using Basta and Inspire has been described (Rausenberger et al., 2011).

The yeast three-hybrid vector, pBridge (Clontech), was modified to replace multiple cloning sites and to remove the N-terminal fusion from the second cloning site. Synthetic oligonucleotides 5'-AAT TGG ATC CAG AAT TCA CTA GTT AAT GCA-3' and 5'-TTA ACT AGT GAA TTC TGG ATC C-3' were annealed and ligated into EcoRI/PstI cut pBridge. Subsequently pBridge was cut with XbaI/BglII and the fragment replaced with a fragment generated by PCR using 5'-ACG TCT CTA GAG CAC ATT CTG CG-3', 5'-ACG TCG GAT CCT TAC CTA GGC TGC AGA GAT CTT GTA TGG ATG GGG GTA ATA G-3', and pBridge as a template, that was cut with XbaI and BamHI. COP1 was amplified by PCR from Arabidopsis total cDNA with 5'-CGC GGA TCC AAA AAT GGA AGA GAT TTC GAC GGA CCC GGT TG-3' and 5'-GGA CTA GTC GCA GCG AGT ACC AGA ACT TTG-3', creating a silent mutation in the internal BamHI site, cut with BamHI/SpeI, and cloned into the BamHI/SpeI sites of modified pBridge. PHYA-NLS as described above, was cut from pBS II KS using BamHI/SpeI, and cloned into the BglII/AvrII sites of modified pBridge. For generation of vectors for PHYB-NLS, the second multiple cloning site of pBridge was alternately modified by cutting XbaI/BglII, and ligating in a new XbaI/BamHI cut fragment generated from pBridge by PCR using 5'-ACG TCT CTA GAG CAC ATT CTG CG-3' and 5'-ACG TCG GAT CCT TAC CTA GGC TGC AGA GAT CTT GTA TGG ATG GGG GTA ATA G-3'. Subsequently full length PHYB, cut with XbaI from pCHF40-PHYB was cloned into the NheI site.

pCGADT7ah-PHYB is a yeast two hybrid vector coding for *PHYB-GAL4 AD*. To obtain it, PHYB coding sequence was amplified from *Pro35S:PHYB-GFP* (Hiltbrunner et al., 2005) using oligos 5'-CCC AAG CTT CTA GAA AAA TGG TTT CCG GAG TCG GG-3'/5'-GGG GTA CCT TAT CTA GAA TAT GGC ATC ATC AGC ATC A-3', cut with XbaI and cloned into the XbaI site of pCGADT7ah (Rausenberger et al., 2011).

The PHYA-, PHYA 1-406-, PHYA 1-617-, and PHYA C323A-binding domain vectors (pD153AH) have been previously described (Hiltbrunner et al., 2006; Rausenberger et al., 2011).

PHYA G727E and PHYA E777K were created by overlap extension PCR using primer pairs 5'-AGA CAC TCT TGT GCG ATA TG-3'/5'-ACA AAA CAC ACC **T**CA ACC ACG TTT T-3' + 5'-AAA ACG TGG TTG **A**GG TGT GTT TTG T-3'/5'-GGC AAG TTG CAG GAA ACA GA-3' (G727E), or 5'-AGA CAC TCT TGT GCG ATA TG-3'/ 5'-TGG ATT CCA CT**T** TGT GCA CCA TC-3' + 5'-GAT GGT GCA CA**A** AGT GGA ATC CA-3'/5'-GGC AAG TTG CAG GAA ACA GA-3' (E777K), cut with AvrII/SpeI and cloned into AvrII/SpeI cut pD153AH-PHYA.

The yeast two-hybrid activation domain vector, pGADT7 (Clontech), was modified using annealed oligonucleotides 5'-TAT GGA TCC CGG GAC TAG TTA AA-3' and 5'-GAT CTT TAA CTA GTC CCG GGA TCC A-3' ligated into NdeI/BamHI cut plasmid to replace the multiple cloning site. FHY1 was amplified by PCR with 5'-CGC GGA TCC AAA AAT GCC TGA AGT GGA AGT G-3' and 5'-ACG TCA CTA GTT TAC AGC ATT AGC GTT GAG AAG T-3', cut with BamHI/SpeI, and cloned into the BamHI/SpeI sites of modified pGADT7. SPA1 was cut from pBS II KS-SPA1 using XbaI and ligated into the SpeI site of modified pGADT7. SPA2/3/4 PCR products were each cut using BamHI/SpeI and cloned into the BamHI/SpeI sites of modified pGADT7.

Deletion constructs of SPA1 were generated by PCR using 5'-ACG TCG CTA GCG GCA TGT TAC TTA AAA GAG CTA TGA AAG G-3' and 5'-ACG TCG CTA GCA ACA AGT TTT AGT AGC TTC ATG TT-3' for SPA1 203-1029, 5'-ACG TCG CTA GCG GCA TGC CTG TTA TGG AAA GAG TAG-3' and 5'-ACG TCG CTA GCA TAC CGA GCA AAT TTG CAC AAC-3' for SPA1 1-698, both cut with NheI and cloned into the SpeI site of modified pGADT7. SPA1 203-529 was generated by PCR using 5'-ACG TCG GAT CCA GGA ATG GTT ACT TAA AAG AGC TAT GAA AG-3'/5'-ACG TCA CTA GTT ATC AAC TCT GAC TTT AGT ATA TC-3' and cloned into the BamHI/SpeI sites of modified pGADT7. SPA1 1-202, 530-1029 and SPA1 1-529, 699-1029 were generated by overlap extension PCR using primer pairs 5'-ACG TCG CTA GCG GCA TGC CTG TTA TGG AAA GAG TAG-3'/5'-CAT CCT CGC ACA ACT GAG AAA AAT TCG AAG-3' + 5'-TTC TCA GTT GTG CGA GGA TGA TTC AGT T-3'/5'-ACG TCG CTA GCA ACA AGT TTT AGT AGC TTC ATG TT-3' and 5'-ACG TCG CTA GCG GCA TGC CTG TTA TGG AAA GAG TAG-3'/5'-GAA CTT GCT TAT CAA CTC TGA CTT TAG T-3' + 5'-AGA GTT GAT AAG CAA GTT CGA AAC CTG TG-3'/5'-ACG TCG CTA GCA ACA AGT TTT AGT AGC TTC ATG TT-3'. Flanking primers as above were used for the second round. Products were cut with NheI and cloned into the SpeI site of modified pGADT7.

The yeast two-hybrid DNA binding domain vector pGBKT7 (Clontech) was modified as above for pGADT7, to replace the multiple cloning site. Full-length SPA1, cut with XbaI as above was cloned into the SpeI site of modified pGBKT7 to obtain pGBKT7-SPA1.

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Light-Activated Phytochrome A and B Interact with Members of the SPA Family to Promote Photomorphogenesis in Arabidopsis by Reorganizing the COP1/SPA Complex

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REVIEW

SPA proteins: SPAnning the gap between visible light and gene expression

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Abstract

Main conclusion **In this review we focus on the role of SPA proteins in light signalling and discuss different aspects, including molecular mechanisms, specificity, and evolution.**

The ability of plants to perceive and respond to their environment is key to their survival under ever-changing conditions. The abiotic factor light is of particular importance for plants. Light provides plants energy for carbon fixation through photosynthesis, but also is a source of information for the adaptation of growth and development to the environment. Cryptochromes and phytochromes are major photoreceptors involved in control of developmental decisions in response to light cues, including seed germination, seedling de-etiolation, and induction of flowering. The SPA protein family acts in complex with the E3 ubiquitin ligase COP1 to target positive regulators of light responses for degradation by the 26S proteasome to suppress photomorphogenic development in darkness. Light-activated cryptochromes and phytochromes both repress the function of COP1, allowing accumulation of positive photomorphogenic factors in light. In this review, we highlight the role of the SPA proteins in this process and discuss recent advances in understanding how SPAs link light-activation of photoreceptors and downstream signaling.

Keywords COP1 · Cryptochrome · Photomorphogenesis · Phytochrome · SPA proteins

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Photoreceptors in plants

Light has a strong impact on the abundance and distribution of plants. Plants not only use light for photosynthesis, but also derive vital information on the conditions in their habitat by monitoring different aspects of light, including intensity (fluence rate), quality (spectral composition), and spatiotemporal patterns. This information is important for plants to adapt growth and development to the requirements to strive in their environment. The enormous plasticity of plants is a very successful strategy in the struggle for life and prerequisite for survival under diverse conditions.

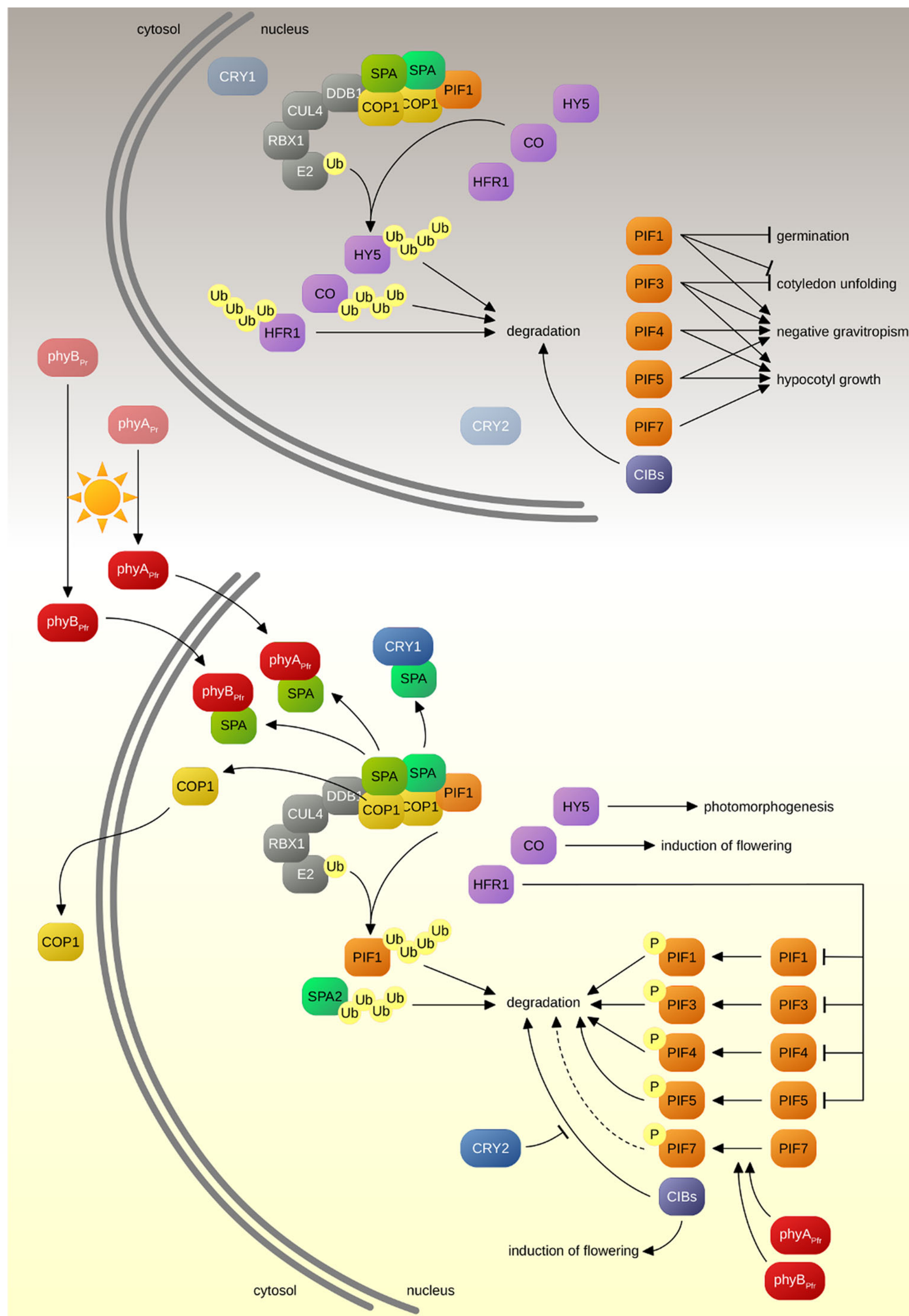
Plants possess several classes of photoreceptors, which are sensitive to different ranges of the light spectrum: UVR8 is a UV-B receptor, phytochromes detect red/far-red, while cryptochromes, phototropins, and ZTL-family proteins perceive blue light (Mancinelli 1994; Chaves et al. 2011; Li et al. 2011; Rizzini et al. 2011; Ito et al. 2012; Tilbrook et al. 2013; Jenkins 2014; Christie et al. 2015; Fankhauser and Christie 2015). Although there is extensive overlap between responses regulated by light of different wavelengths, such as seedling de-etiolation by either red or blue light, it is possible to define subsets of responses mediated by specific classes of photoreceptors (Peschke and Kretsch 2011). Acclimation of plants to UV-B is

dependent on UVR8 (Favory et al. 2009). The ZTL-family photoreceptors are involved in entrainment of the circadian clock and induction of flowering (Ito et al. 2012). Phototropins optimize photosynthesis by controlling phototropic growth, chloroplast positioning, and stomatal opening and closure (Takemiya et al. 2005; Christie 2007). Together, both cryptochromes and phytochromes play a general role in regulation of growth and development, including seed germination, de-etiolation, input to the circadian clock, flowering time, and responses to shade and competition (Chaves et al. 2011; Li et al. 2011; Casal 2012, 2013; Wang and Wang 2015). There are two cryptochromes (CRY1 and CRY2) in both monocots and dicots. CRY2 is unstable in light and particularly important under low light conditions, whereas CRY1 is light-stable and active over a large range of fluence rates (Lin et al. 1998; Chaves et al. 2011). The phytochrome family in *Arabidopsis* consists of five members (phyA to phyE) (Mathews 2006; Li et al. 2011; Wang and Wang 2015). Of these phytochromes, phyA, phyB, and phyC are conserved in all monocots and dicots investigated so far (Mathews 2006). phyA is required for responses to very low light intensities, short light pulses, and light conditions with a high far-red light content (Yanovsky et al. 1995; Botto et al. 1996). It is of particular relevance during de-etiolation and at the seedlings stage and plays a less dominant role in adult plants, where phyA contributes to regulation of growth habit and flowering (Whitelam et al. 1993; Johnson et al. 1994; Franklin et al. 2003b). In contrast, phyB is the primary phytochrome at the adult stage (Sharrock and Clack 2002; Li et al. 2011). It is activated by red light and inactivated by far-red light and as such ideally suited for measuring the red:far-red light ratio, which is an indicator of canopy shade and competition by neighboring plants (Li et al. 2011; Casal 2012, 2013). The remaining three members of the phytochrome family of *Arabidopsis* have more subtle functions. In the absence of phyA or phyB, the effects of phyC/D/E on regulation of plant architecture and induction of both germination and flowering can be observed (Hennig et al. 2002; Franklin et al. 2003a, b; Monte et al. 2003). However, phyC has a more noticeable function in monocots, where it contributes to induction of flowering in wheat and barley, though this function may be dependent on phyB (Nishida et al. 2013; Chen et al. 2014).

Skotomorphogenesis and photomorphogenesis

Germinating seedlings employ very different developmental programs depending on the availability of light: skotomorphogenesis in the dark and photomorphogenesis in the light (Kami et al. 2010; Li et al. 2011). Skotomorphogenic growth depends on the energy stored in seeds and is characterized by strong hypocotyl elongation, closed cotyledons, and an apical hook that protects the shoot apex from mechanical damage. Seedlings germinating from seeds in the soil use this strategy to reach the soil surface, where light—primarily perceived by cryptochromes and phytochromes—promotes de-etiolation, the transition from skotomorphogenesis to photomorphogenesis. Photomorphogenic development includes inhibition of hypocotyl elongation, unfolding of the apical hook and cotyledons, chlorophyll biosynthesis, and assembly of the photosynthetic apparatus, and eventually leads to photosynthetically competent seedlings (Kami et al. 2010). Two main signal transduction pathways link light perception by phytochromes and cryptochromes to differential regulation of several hundreds of genes responsible for the de-etiolation response (Ma et al. 2001; Jiao et al. 2005; Peschke and Kretsch 2011). The PHYTOCHROME INTERACTING FACTORs (PIFs), CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), and members of the SUPPRESSOR OF *phyA-105* (SPA) family are key components of these pathways and repress photomorphogenesis (Fig. 1) (Deng et al. 1991, 1992; Hoecker et al. 1998, 1999; Ni et al. 1998; Laubinger et al. 2004; Leivar and Quail 2011). It is worth mentioning that the PIF and COP1/SPA dependent pathways are not entirely independent as at least one of the PIFs, PIF3, does not accumulate in the absence of COP1 and SPAs (Bauer et al. 2004; Leivar et al. 2008; Dong et al. 2014). Moreover, mutual regulation between COP1/SPA and PIF1 (=PIF3-LIKE 5, PIL5), another member of the PIF family, has been demonstrated (Xu et al. 2014; Zhu et al. 2015).

Fig. 1 Model for phytochrome and cryptochrome mediated light ▶ responses. In dark-grown seedlings and plants (*upper half*), the CUL4-DDB1$^{COP1/SPA}$ E3 ligase complex promotes the degradation of transcription factors involved in light responses (HY5, HFR1, CO; *violet*). The CUL4/DDB1/RBX1 E3 ligase core (*gray*) binds COP1/SPA and E2 ubiquitin conjugating enzymes, which provide the ubiquitin moieties transferred to target proteins recognized by COP1/SPA. In parallel, photomorphogenesis and other light responses are also inhibited by PIFs (*orange*); PIF1 also enhances the activity of the COP1/SPA complex. CIBs are degraded in dark-grown plants. In light-grown plants (*lower half*), active phytochromes (phyA$_{Pfr}$ and phyB$_{Pfr}$) are transported into the nucleus. Light-activated phyA, phyB, and CRY1 down-regulate the activity of the CUL4-DDB1$^{COP1/SPA}$ E3 ligase complex by promoting nuclear depletion of COP1 and disrupting the direct interaction of COP1 and SPAs. In addition, phytochromes target PIFs for degradation (*dashed arrow* for PIF7 indicates that the degradation of PIF7 is much slower than for other PIFs) and also induce COP1-mediated degradation of PIF1, SPA2, and possibly SPA1. Light-activated CRY2 stabilizes CIBs and, thereby, promotes induction of flowering. For clarity, only a representative subset of COP1/SPA complex targets and PIFs are shown, and the model does not cover all known light signaling events. Inactive cryptochromes (CRY1 and CRY2) are shown in *pale blue*; inactive phytochromes (phyA$_{Pr}$ and phyB$_{Pr}$) are shown in *pale red*. Active cryptochromes and phytochromes are shown in *blue* and *red*, respectively. *Ub* ubiquitin moieties attached to COP1/SPA target proteins. *P* phosphate groups attached to PIF proteins



The PIFs, including PIF1 and PIF3 through PIF8, form a subgroup of the bHLH transcription factors (Leivar and Quail 2011; Leivar and Monte 2014). They have only partially redundant functions and contribute differently to the various aspects of photomorphogenesis (Fig. 1). PIF1, for instance, is an important inhibitor of seed germination but plays a less prominent role in regulation of growth (Oh et al. 2004; Shin et al. 2009). PIF1, PIF3, PIF4, and PIF5 promote hypocotyl elongation in dark-grown seedlings, and PIF4, PIF5, and PIF7 are of particular importance for auxin-mediated growth responses to high temperature and canopy shade (Leivar et al. 2008; Koini et al. 2009; Shin et al. 2009; Franklin et al. 2011; Sun et al. 2012; Li et al. 2012; Hornitschek et al. 2012; de Wit et al. 2014). Phytochromes translocate from the cytosol into the nucleus in light-grown plants and inactivate PIFs by inhibiting their

binding to target promoters and targeting them for degradation by the 26S proteasome (Fig. 1) (Shen et al. 2005; Oh et al. 2006; Al-Sady et al. 2006; Li et al. 2012; Park et al. 2012; Klose et al. 2015). Reduced levels of different PIFs in light-grown seedlings induce germination and promote photomorphogenic development.

Photomorphogenesis is also repressed by COP1 and SPAs. They are part of an E3 ubiquitin ligase complex and suppress photomorphogenic development in dark-grown seedlings by promoting the degradation of positive regulators of photomorphogenesis (Fig. 1) (Deng et al. 1991, 1992; Laubinger et al. 2004). While *COP1* is a single copy gene in *Arabidopsis*, a gene family with four members encodes for SPA proteins (*SPA1*, *SPA2*, *SPA3*, and *SPA4*). The different SPAs are partially redundant but also have specific functions. In summary, SPA3 and SPA4 contribute to regulation of growth and development in de-etiolated seedlings and, in particular, in adult plants, whereas SPA1 and SPA2 are primarily or exclusively active in seedlings (Fig. 2) (Laubinger and Hoecker 2003; Laubinger et al. 2004). SPA2 is sufficient for suppression of photomorphogenesis in etiolated seedlings but appears not to have any function at later stages in development (Laubinger et al. 2004; Balcerowicz et al. 2011). SPA1 plays a dominant role in etiolated and de-etiolated seedlings and in regulation of flowering induction, while other responses in adult plants are not or only slightly affected in the absence of SPA1 (Laubinger et al. 2004, 2006; Ishikawa et al. 2006).

Mutants with reduced COP1 activity or containing defects in several of the *PIF* or *SPA* genes are unable to fully suppress photomorphogenic development in darkness (Hoecker et al. 1998, 1999; Laubinger and Hoecker 2003; Ordoñez-Herrera et al. 2015). Such mutants are referred to as *constitutively photomorphogenic* and have short hypocotyls and open cotyledons in the dark (Fig. 3) and also resemble light-grown plants at the transcriptome level (Ma et al. 2002; Laubinger et al. 2004; Leivar et al. 2008, 2009; Shin et al. 2009).

COP1/SPA as negative regulator of photomorphogenesis

COP1 and SPA proteins are part of a >700 kDa complex, consisting of the CULLIN 4 (CUL4)/DAMAGED DNA BINDING 1 (DDB1)/RING BOX 1 (RBX1) E3 ligase core complex and the COP1/SPA complex (Fig. 1) (Saijo et al. 2003; Lau and Deng 2012). COP1/SPA acts as substrate receptor in this CUL4-DDB1$^{COP1/SPA}$ E3 ligase complex, which we will refer to as the COP1/SPA complex. For details on the CUL4/DDB1/RBX1 core complex and on COP1, we direct readers to excellent reviews specifically focusing on these topics (Smirnova et al. 2011, 2012; Lau and Deng 2012; Huang et al. 2014). Here, we focus on the SPA proteins and their function in linking photoreceptor activation and inhibition of COP1 function.

The COP1/SPA complex targets positive regulators of photomorphogenesis, including ELONGATED HYPOCOTYL 5 (HY5) and LONG HYPOCOTYL IN FAR-RED 1 (HFR1) for degradation by the 26S proteasome (Table 1) (Osterlund et al. 2000; Holm et al. 2002; Seo et al. 2003; Duek et al. 2004; Jang et al. 2005; Yang et al. 2005a, b). Through this mechanism, the COP1/SPA complex can suppress photomorphogenic development in the dark, but also prevent exaggerated photomorphogenesis in light-grown seedlings and adult plants (Deng et al. 1991; McNellis et al. 1994; Hoecker et al. 1998; Laubinger and Hoecker 2003; Laubinger et al. 2004). Moreover, COP1 and SPAs are required for the shade avoidance response

	spa1	*spa2*	*spa3*	*spa4*	*spa12*	*spa34*	*spa123*	*spa124*	*spa134*	*spa234*	*spa1234*
de-etiolation in the dark	1, 2, 3, 4	3, 5	6	5, 6	3, 5	4, 5, 6	3, 5	3, 5	3, 4, 5	4, 5	3, 4, 5
reduced hypocotyl growth in far-red light	1, 2, 3, 4	3, 5	6	5, 6	3	4, 5, 6	3, 5	3, 5	3, 4, 5	4, 5	5
reduced hypocotyl growth in red light	1, 2, 3, 4	3, 5	6	5, 6	3	4, 6	3	3	3, 4	4	ND
reduced hypocotyl growth in blue light	3, 4	3, 5	6	5, 6	3	4, 6	3	3	3, 4	4	ND
increased anthocyanin levels in the dark	1, 2, 3	3	ND	ND	3	6	ND	ND	ND	ND	5
increased anthocyanin levels in light	1, 2, 3, 4	3	ND	ND	3	6	ND	ND	ND	ND	5
petiole length reduced to <70% of WT	3	3	3	3	3	3, 4	3	3	3, 4	4	ND
leaf growth reduced to <70% of WT	3	3	3	3	3	3, 4	3	3	3, 4	4	ND
dwarf phenotype (<70% of rosette diameter of WT)	3	3	3	3	3, 5	3, 4, 5	3, 5	3, 5	3, 4, 5	4, 5	3, 5
dwarf phenotype (<70% of height of WT)	ND	ND	ND	ND	3	3	3	3	3	ND	3
early flowering in short day	7, 8	7	7	7	ND	ND	5	5	5, 7	5, 7	5
no increase of hypocotyl growth in low red:far-red light	9	9	9	9	9	9	9	9	9	9	9

no yes

Fig. 2 Phenotypes of *spa* mutants. The table summarizes different phenotypes of *spa* single and higher order mutants described in the literature. *Blue* indicates that the respective mutant exhibits the phentoype; *red* indicates that this is not the case (i.e., the mutant has a WT phenotype). *Numbers* one to nine give the references: *1* Hoecker et al. (1998); *2* Baumgardt et al. (2002); *3* Laubinger et al. (2004); *4* Fittinghoff et al. (2006); *5* Ordoñez-Herrera et al. (2015); *6* Laubinger and Hoecker (2003); *7* Laubinger et al. (2006); *8* Ishikawa et al. (2006); *9* Rolauffs et al. (2012)

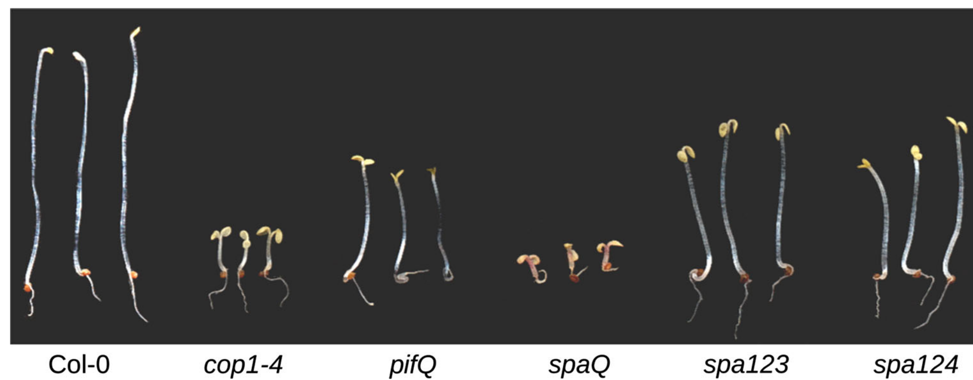


Fig. 3 *Constitutively photomorphogenic* (*cop*) phenotype. Wild type (Col-0), *cop1-4*, *pifQ* (=*pif1-1 pif3-3 pif4-2 pif5-3*), *spaQ* (*spa1-100 spa2-2 spa3-1 spa4-3*), *spa123* (*spa1-7 spa2-1 spa3-1*), and *spa124* (*spa1-3 spa2-1 spa4-1*) seedlings were grown in darkness for 4 days, following germination induction (McNellis et al. 1994; Laubinger et al. 2004, 2006; Leivar et al. 2008; Ordoñez-Herrera et al. 2015)

and neighbor detection in light-grown plants, which depend on the detection of the red:far-red light ratio in the ambient environment (McNellis et al. 1994; Rolauffs et al. 2012; Pacín et al. 2013). In addition, photoperiodic flowering is controlled by the COP1/SPA complex, which targets for degradation several components regulating this response, including CONSTANS (CO), GIGANTEA (GI), and EARLY FLOWERING 3 (ELF3) (Table 1) (Laubinger et al. 2006; Liu et al. 2008b; Jang et al. 2008, 2015; Yu et al. 2008). Upon activation by light, both phytochromes and cryptochromes downregulate the activity of the COP1/SPA complex, allowing positive regulators of light responses to accumulate (Fig. 1) (Lau and Deng 2012). Thus, COP1 and SPAs regulate plant growth and development throughout the entire life cycle.

Interestingly, the COP1/SPA complex has also been implicated in the degradation of CRY2, phyA, and phyB, providing an additional pathway to regulate light responses. CRY2 is partially stabilized in lines lacking either functional COP1 or several SPAs, indicating that the COP1/SPA complex is involved in CRY2 protein turnover (Shalitin et al. 2002; Weidler et al. 2012). It appears unlikely that the CUL4-DDB1$^{COP1/SPA}$ E3 ligase complex plays a direct role in phyA and phyB degradation. Under standard growth conditions (in the absence of metabolisable sugar), turnover of phyA depends on SPAs and CUL1 but not on COP1 and CUL4 (Debrieux et al. 2013). Thus, SPAs may have a function in phyA turnover that is independent of COP1 and the CUL4-DDB1$^{COP1/SPA}$ complex. Interestingly, in the presence of metabolisable sugar, COP1 enhances phyA degradation, suggesting that the stability of phyA is regulated not only by photomorphogenic light but also by the energy status of the plant, which involves a COP1-dependent mechanism. *In vitro* assays suggested that COP1 ubiquitinates phyB and that the efficiency of ubiquitination is enhanced by PIFs (Jang et al. 2010). phyB degradation is indeed strongly inhibited in higher order *pif* mutants; however, it is yet to be shown that COP1 is directly responsible for phyB ubiquitination in plants (Jang et al. 2010; Leivar et al. 2012). It has recently been shown that LIGHT-RESPONSE BRIC-A-BRACK/TRAMTRACK/BROAD COMPLEX proteins (LRBs) are required for light-induced phyB (and PIF3) ubiquitination and protein turnover (Ni et al. 2014). LRBs are components of CUL3/LRB E3 ligase complexes and, similar to COP1/SPA in the CUL4-DDB1$^{COP1/SPA}$ complex, responsible for substrate recognition (Hotton and Callis 2008; Lau and Deng 2012; Ni et al. 2014). LRBs bind to PIF3, and subsequently also target PIF3-bound phyB for codegradation (Ni et al. 2014). Because PIF3 protein levels are reduced in a *cop1* mutant background (Bauer et al. 2004; Dong et al. 2014), the CUL3/LRB E3 ligase-dependent degradation of PIF3/phyB may be inhibited in the absence of functional COP1; in this case, COP1 would only indirectly contribute to phyB protein turnover and not be part of the E3 ubiquitin ligase that ubiquitinates phyB *in planta*.

COP1/SPA complex as a hub of environmental and hormone signaling

The bZIP transcription factor HY5, the first COP1/SPA complex target to be identified, does not only play a central role in light signaling but is also involved in responses to hormones and temperature (Osterlund et al. 2000; Lau and Deng 2010; Catalá et al. 2011). Gibberellins and cytokinin both regulate HY5 at the protein level (Vandenbussche et al. 2007; Alabadí et al. 2008). Because this regulation is impaired in a *cop1* mutant with reduced activity, a role of COP1 in integration of light, gibberellin, and cytokinin signals has been proposed (Vandenbussche et al. 2007; Alabadí et al. 2008). Besides the function in light and hormone signaling, HY5 also promotes cold acclimation. This process depends on cold-induced inactivation of COP1

Table 1 COP1 target proteins

Targets of COP1		SPA dependent	Function	Reference
BBX22/LZF/STH3	AT1G78600	ND	Positive regulator of photomorphogenesis	Chang et al. (2011)
BBX24/STO	AT1G06040	ND	Negative Regulator of photomorphogenesis; positive regulator of flowering	Indorf et al. (2007), Li et al. (2014)
BBX25/STH	AT2G31380	ND	Negative regulator of photomorphogenesis	Gangappa et al. (2013)
CO/BBX1	AT5G15840	Yes	Positive regulator of flowering	Jang et al. (2008), Laubinger et al. (2006)
CRY2	AT1G04400	Yes	Blue light receptor	Shalitin et al. (2002), Weidler et al. (2012)
ELF3	AT2G25930	ND	Negative regulator of flowering; circadian clock	Yu et al. (2008)
GATA2	AT2G45050	ND	Positive regulator of photomorphogenesis; brassinosteroid signaling	Luo et al. (2010)
GI[a]	AT1G22770	ND	Positive regulator of photomorphogenesis and flowering; circadian clock	Yu et al. (2008)
HFR1	AT1G02340	Yes	Positive regulator of photomorphogenesis; negative regulator of shade avoidance response	Yang et al. (2005a), Yang et al. (2005b)
HY5	AT5G11260	Yes	Positive regulator of photomorphogenesis	Osterlund et al. (2000), Yang and Wang (2006)
HYH	AT3G17609	ND	Positive regulator of photomorphogenesis	Holm et al. (2002)
LAF1	AT4G25560	ND	Positive regulator of photomorphogenesis	Seo et al. (2003)
PAP2/MYB90	AT1G66390	ND	Regulation of anthocyanin biosynthesis	Maier et al. (2013)
phyA[b]	AT1G09570	Yes	Red/far-red/blue light receptor	Seo et al. (2004), Debrieux et al. (2013)
PIF1/PIL5	AT2G20180	Yes	Negative regulator of seed germination and photomorphogenesis	Zhu et al. (2015)
SPA2	AT4G11110	ND[c]	Component of COP1/SPA complex	Chen et al. (2015)

Summary of proteins targeted for degradation by COP1. The third column indicates if degradation of the respective COP1 target protein is abolished or reduced in *spa* single or *spa* higher order mutants; *ND* not determined

[a] Interaction with COPI depends on ELF3

[b] Only in the presence of metabolisable sugar

[c] Not dependent on SPA1, SPA3, and SPA4

and concomitant stabilization of HY5 (Catalá et al. 2011). Thus, COP1 is a central hub for the integration of environmental and developmental signals (Lau and Deng 2010; Huang et al. 2014). The requirement of SPA proteins for proper control of protein stability of HY5 and other factors, including HFR1 and CO, in response to light perceived by cryptochromes and phytochromes is well established and discussed in more detail in the following chapters (Yang et al. 2005a; Laubinger et al. 2006; Lian et al. 2011). In contrast, it is still an open question if SPAs are required for gibberellin, cytokinin, and temperature regulation, and it has not been tested if HY5 protein turnover in response to these stimuli depends on SPA proteins. It is reasonable to assume that COP1 has SPA independent functions, given that an *Arabidopsis* mutant lacking any functional SPAs (*spa1, spa2, spa3, spa4*; also referred to as *spaQ*) is viable but strong *cop1* mutant alleles are lethal (McNellis et al. 1994; Laubinger et al. 2004; Ordoñez-Herrera et al. 2015). Thus, SPAs may have a specific function in phytochrome and cryptochrome downstream signaling and link COP1 to perception of light in the visible spectrum. In contrast, there is currently no evidence for a role of SPAs in regulation of COP1's E3 ubiquitin ligase activity in response to stimuli other than visible light.

Role of COP1 in UV-B-induced photomorphogenesis

Responses to photomorphogenic UV-B light require the photoreceptor UVR8 and the downstream signaling component COP1 but do not depend on the SPA proteins (Oravecz et al. 2006; Favory et al. 2009; Rizzini et al. 2011)—although it is controversially discussed if SPAs may enhance responses by UVR8 (Huang et al. 2013). Another intriguing difference between perception of light

in the visible and the UV-B range of the spectrum is that COP1 functions as a negative regulator of light responses downstream of cryptochromes and phytochromes, whereas it acts as a positive regulator in photomorphogenic UV-B light perceived by UVR8 (Oravecz et al. 2006). UVR8 dimers monomerise in response to UV-B light (Rizzini et al. 2011; Christie et al. 2012; Wu et al. 2012; Zeng et al. 2015), and it has been suggested that binding of UVR8 monomers to COP1 disconnects COP1/SPA from the CUL4/DDB1 E3 ubiquitin ligase core complex to establish a unique UVR8/COP1/SPA complex that stabilizes HY5 instead of promoting its degradation (Huang et al. 2013). It is important to note that UV-B activated UVR8 does not affect binding of COP1 to SPA1 (and possibly other SPAs) (Huang et al. 2013; Heijde et al. 2013), while cryptochromes and phytochromes are thought to control the COP1/SPA activity at least partially by regulating the interaction of COP1 and SPAs (Lian et al. 2011; Liu et al. 2011a; Zuo et al. 2011; Sheerin et al. 2015; Lu et al. 2015).

Many paths lead to COP1/SPA complex inactivation

The long-standing model how visible light regulates COP1 dates from the pre-SPA-era, when it was unknown that COP1 requires the SPA proteins for E3 ubiquitin ligase activity. Soon after identification of COP1 as a key factor in repression of photomorphogenesis (Deng et al. 1991, 1992), it was shown that nucleocytoplasmic partitioning of GUS-COP1 fusion proteins is regulated by visible light and that phytochromes and cryptochromes are necessary to promote depletion of GUS-COP1 from the nucleus in a wavelength-specific manner (von Arnim and Deng 1994; von Arnim et al. 1997; Osterlund and Deng 1998). Based on these findings, it has been proposed that nuclear depletion of COP1 is responsible for the stabilization and accumulation of positive regulators of photomorphogenesis in light-grown seedlings. Yet, inhibition of hypocotyl elongation upon exposure to light occurs with a lag phase of 10–90 min, whereas relocation of GUS-COP1 from the nucleus into the cytosol is much slower and requires 12 h or even longer (von Arnim and Deng 1994). Therefore, it has been hypothesised that the kinetics for GUS-COP1 nuclear depletion might be different from that of endogenous COP1—even though GUS-COP1 complements the strong *cop1-5* mutant allele (von Arnim et al. 1997)—or that redistribution of COP1 is only required to maintain fate decisions but not to induce such commitments (von Arnim and Deng 1994; von Arnim et al. 1997). More recent work has shown that nuclear depletion of YFP-COP1 is indeed faster than of GUS-COP1 (Pacín et al. 2014), suggesting that the size of the COP1 (fusion) protein (GUS-COP1: 146 kDa, YFP-COP1: 104 kDa, COP1: 76 kDa) affects the transport kinetics, and that nuclear depletion of untagged COP1 might be even faster than of YFP-COP1. However, it is still questionable if this is fast enough to explain very rapid responses, such as the regulation of early light response genes (Peschke and Kretsch 2011; Rausenberger et al. 2011). Moreover, YFP-COP1 reaccumulates in the nucleus in light-grown seedlings in response to simulated shade (Pacín et al. 2013), and it has been shown that phyA counteracts shade induced hypocotyl elongation (Yanovsky et al. 1995; Smith et al. 1997; Sellaro et al. 2010), suggesting that phyA downstream signaling is active even in the presence of nuclear localized COP1. In addition, UV-B supplemented to white light promotes UVR8 and COP1 nuclear accumulation and regulates gene expression in a strictly COP1-dependent manner (Oravecz et al. 2006; Kaiserli and Jenkins 2007; Favory et al. 2009). Thus, it is reasonable to assume that under natural light conditions, which include UV-B light, COP1 is present in the nucleus. This indicates that alternative mechanisms exist for the regulation of COP1/SPA activity that do not rely on COP1 nuclear depletion.

Work in the past few years suggests that at least two alternative mechanisms for COP1/SPA inactivation exist, relying on the direct regulation of COP1/SPA complex stability by light-activated cryptochromes and phytochromes and on light-regulation of SPA protein turnover (Fig. 1). Light-activated cryptochromes (CRY1 and CRY2) and phytochromes (phyA and phyB) bind to specific members of the SPA family (Fig. 4 and Suppl. Figure 1). Interaction has been confirmed for phyA and SPA1/SPA2, phyB and SPA1, as well as CRY1 and all four SPAs (Lian et al. 2011; Liu et al. 2011b; Zheng et al. 2013; Sheerin et al. 2015; Lu et al. 2015; Chen et al. 2015). Data on the interaction of CRY2 and SPAs are partially contradictory; different reports agree on that CRY2 interacts with SPA1 and SPA4, but there is disagreement regarding the interaction with SPA2/SPA3 (Zuo et al. 2011; Weidler et al. 2012; Chen et al. 2015). SPA proteins consist of a kinase-like domain, a coiled-coil domain, and a WD40 domain; SPA1 and SPA2, in addition, contain an N-terminal extension of roughly 300 amino acid residues (Hoecker et al. 1999; Zhu et al. 2008). Analysis of a wide range of SPA deletion constructs shows that there is no common photoreceptor binding motif or domain in SPAs (Fig. 4 and Suppl. Figure 1) (Lian et al. 2011; Liu et al. 2011a; Zuo et al. 2011; Zheng et al. 2013; Sheerin et al. 2015; Lu et al. 2015). phyA requires the kinase-like domain for binding to SPA1 (Sheerin et al. 2015), phyB possibly interacts with the coiled-coil domain (Zheng et al. 2013), and CRY2 depends on the kinase-like domain and/or the N-terminal extension for binding to SPAs—although the N-terminal extension appears less likely as binding site, because it is

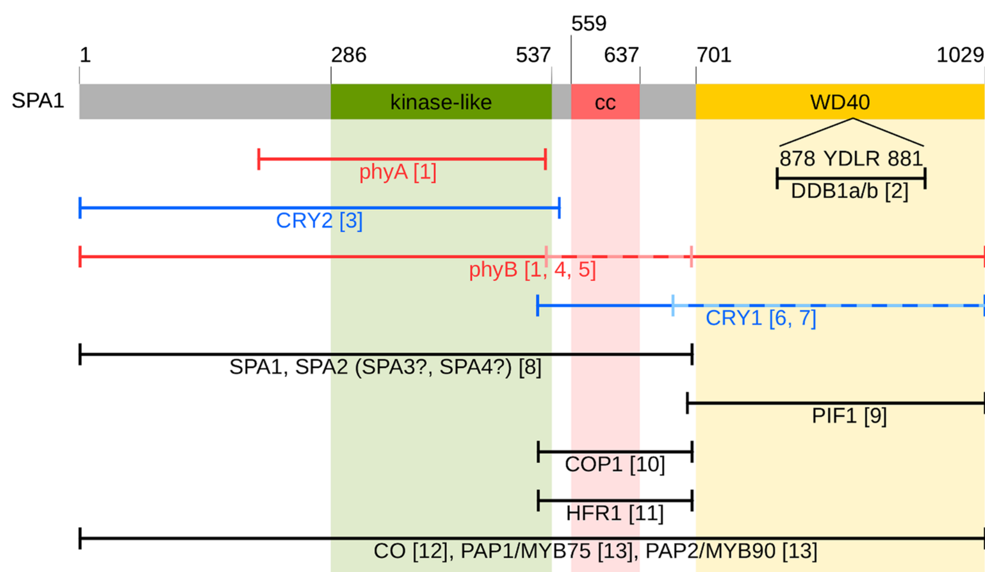


Fig. 4 Domains of SPA1 responsible for binding to different SPA1 interacting proteins. The SPA1 protein is shown schematically (kinase-like domain in *green*, coiled-coil domain in *red,* and WD40 domain in *yellow*); numbers indicate amino acid positions. *Bars* for different SPA1 interacting proteins indicate the SPA1 domains required for the interaction. For binding of DDB1a/b, the YDLR amino acid motif is required. *Red* and *blue bars* indicate *red* and *blue light* dependent interaction; the *dashed lines* for phyB and CRY1 show SPA1 fragments that bind independent of light; *black bars* indicate light-independent interactions. *Numbers* 1–13 give the references: *1* = Sheerin et al. (2015); *2* = Chen et al. (2010); *3* = Zuo et al. (2011); *4* = Lu et al. (2015); *5* = Zheng et al. (2013); *6* = Lian et al. (2011); *7* = Liu et al. (2011a); *8* = Zhu et al. (2008); *9* = Zhu et al. (2015); *10* = Hoecker and Quail (2001); *11* = Yang et al. (2005a); *12* = Laubinger et al. (2006); *13* = Maier et al. (2013)

not conserved among the SPA protein family (Fig. 4) (Zuo et al. 2011). CRY1 binds to the WD40 domain but possibly requires the coiled-coil domain for regulation by blue light (Lian et al. 2011; Liu et al. 2011a). Binding of phyA, phyB, and CRY1 to SPAs inhibits the interaction of SPAs and COP1, and at least phyB and CRY1 promote the dissociation of the complex (Lian et al. 2011; Liu et al. 2011a; Sheerin et al. 2015; Lu et al. 2015). COP1 also interacts with phyA; therefore, binding of phyA to SPAs may not result in full dissociation of the COP1/SPA complex but remodel the complex, so that the direct interaction between COP1 and SPAs is lost (Viczián et al. 2012; Sheerin et al. 2015). Because binding of SPAs to COP1 is essential for COP1's E3 ubiquitin ligase activity (Saijo et al. 2003; Zhu et al. 2008), disruption of the COP1–SPA interaction by light-activated phyA, phyB, and CRY1 would inactivate the complex and stabilize proteins targeted for degradation by the COP1/SPA complex (Lian et al. 2011; Liu et al. 2011a; Sheerin et al. 2015; Lu et al. 2015). Interestingly, binding of CRY2 to SPA1 strengthens the interaction of COP1 and CRY2, but, nevertheless, suppresses the activity of the COP1/SPA complex (Zuo et al. 2011). The common theme in sensing visible light through the COP1/SPA complex is, therefore, the direct binding of phytochromes and cryptochromes to SPA proteins. This also distinguishes phytochromes and cryptochromes from the UV-B sensing UVR8, which does not directly interact with SPAs (Huang et al. 2013; Heijde et al. 2013). However, based on the different binding sites and effects on COP1/SPA complex stability, phyA, phyB, CRY1, and CRY2 appear to use at least partially different mechanisms to downregulate COP1's E3 ubiquitin ligase activity. Moreover, it is still unknown if the remaining phytochromes (phyC, phyD, phyE) interact with SPAs and, if yes, if and how they affect the COP1–SPA interaction.

The regulation of the stability of SPA proteins is another option to control the activity of the COP1/SPA complex. SPA1 and SPA2 are light labile and rapidly degraded in seedlings exposed to red, far-red, or blue light (Balcerowicz et al. 2011; Chen et al. 2015). The turnover of SPA2 in far-red light and low fluence red light depends on phyA, while phyB is sufficient for SPA2 degradation in high fluence red light (Chen et al. 2015). These requirements are not unexpected due to the physiological activity of phyA and phyB under these light conditions. However, it is remarkable that normal SPA2 turnover in blue light cannot be induced by cryptochromes, the main blue light receptors, and instead requires phyA. Consistent with the dominant role of phyA in regulation of SPA2 turnover, the strong hypersensitivity of the *spa1 spa3 spa4* triple mutant toward far-red, blue, and weak read light is fully dependent on phyA (Chen et al. 2015).

SPA1 and SPA2 protein levels are increased in dark-grown *cop1-6* seedlings (Zhu et al. 2008), and their degradation is

blocked by the proteasome inhibitor MG132 (Balcerowicz et al. 2011). Given that COP1 is stable in light (Zhu et al. 2008; Balcerowicz et al. 2011), this points to a possible function of COP1 in SPA1/SPA2 protein turnover. Recent data indeed show that COP1 is required for normal turnover of SPA2 (Chen et al. 2015). Interestingly, SPA2 degradation was hardly reduced in *spa1 spa3 spa4* mutant background, suggesting that either COP1-mediated SPA2 turnover is independent of SPAs or that SPA2, in complex with COP1, can promote its own targeting for degradation (Chen et al. 2015). If binding of phyA to SPA2 is required for SPA2 degradation, is currently unknown, although such a requirement is not unlikely. Thus, recruitment of phyA into the COP1/SPA complex may not simply inactivate the complex but rather change its substrate specificity from HY5, HFR1, and other COP1/SPA substrates of etiolated seedlings to SPA1 and SPA2 and target them for degradation in light-grown plants. SPA2 turnover is extremely fast and occurs within 5–15 min after exposure to light, which is consistent with the induction kinetics of very rapid light response genes (Balcerowicz et al. 2011; Peschke and Kretsch 2011; Rausenberger et al. 2011; Chen et al. 2015).

Despite the direct link of SPAs to COP1, nucleocytoplasmic shuttling of SPAs has not been described so far, and SPAs are considered constitutively nuclear localized proteins (Hoecker et al. 1999; Laubinger et al. 2006; Zhu et al. 2008; Lian et al. 2011; Liu et al. 2011a; Zuo et al. 2011; Sheerin et al. 2015; Lu et al. 2015). Yet, such regulation of SPA nucleocytoplasmic shuttling may hypothetically provide a third mechanism to regulate the activity of the COP1/SPA complex and, thus, could be beneficial to investigate.

Interaction of COP1/SPA and PIF signaling pathways

HFR1 suppresses the activity of several PIFs by forming inactive HFR1/PIF heterodimers, which are unable to bind promoters of PIF target genes (Fig. 1) (Hornitschek et al. 2009; Shi et al. 2013; Bou-Torrent et al. 2015). The COP1/SPA complex regulates the turnover of HFR1 (Duek et al. 2004; Jang et al. 2005; Yang et al. 2005b, a) and, thereby, also controls the activity of these PIFs (Lorrain et al. 2009). In addition to this indirect regulation, it has recently been shown that PIF1 interacts with COP1 and SPA1 (Fig. 1) (Xu et al. 2014). COP1 and, in particular, SPAs promote the phyA-mediated rapid degradation of PIF1 in response to short light treatments (Zhu et al. 2015). Even in *spa1* and *spa2* single mutants, a clear stabilization of PIF1 can be observed (Zhu et al. 2015). This is in strong contrast to SPA2, which either is sufficient for its own degradation or does not depend on SPAs at all for COP1 dependent turnover (Chen et al. 2015), suggesting that the mechanisms of COP1 regulated degradation of PIF1 and SPA2 are at least partially different.

PIF1 interacts with COP1/SPA even in dark-grown plants. However, under these conditions, it is not targeted for degradation and instead works as a positive regulator of the COP1/SPA complex to increase its E3 ubiquitin ligase activity toward HY5 (Xu et al. 2014). Binding of PIF1 to COP1 requires the phyB binding motif of PIF1 (APB) (Xu et al. 2014), while the phyA binding motif (APA) mediates the interaction with SPA1 (Zhu et al. 2015). Thus, PIF1 may work as a "molecular clamp" that ties together COP1 and SPA1, and thereby stabilizes the complex (Fig. 1).

It is worth noting that PIF1 is quite different from other members of the PIF family in many aspects, and it will be important to clarify, if COP1/SPA affects the stability of other PIFs as well. At least PIF3 does not accumulate in dark-grown *cop1-4* and *spa1 spa2 spa3* mutant seedlings (Bauer et al. 2004; Leivar et al. 2008; Dong et al. 2014), whereas PIF1 protein levels in dark-grown *cop1-4* seedlings are only slightly lower, and in *spaQ* seedlings even higher than in the wild type (Zhu et al. 2015). This suggests that PIF1 and PIF3 are differently regulated by COP1 and SPAs. It is interesting that germination of the *cop1-4* mutant still requires light (Deng et al. 1991), whereas the *pif1 pif3 pif4 pif5* (also referred to as *pifQ*) mutant that lacks functional PIF1, PIF3, PIF4, and PIF5 germinates in the dark (Leivar et al. 2008; Shin et al. 2009). PIF4 and PIF5 are more closely related to PIF3 than to PIF1 (Leivar and Quail 2011); therefore, regulation of PIF4/PIF5 by COP1/SPA may be similar to PIF3. If so, *cop1-4* seedlings may not accumulate any PIFs with exception of PIF1. Since PIF1 is the only PIF required for suppression of germination in the dark (Shin et al. 2009), *pifQ* but not *cop1-4* seedlings would germinate independently of light. Thus, it seems possible that PIF1 acquired a mechanisms for the regulation by (and of) COP1/SPA that is unique among the members of the PIF family.

The important conclusion from the recent findings on COP1 regulated turnover of PIF1, SPA2, and possibly SPA1, is that light changes the substrate specificity of the COP1/SPA complex instead of simply inactivating it (Fig. 1). The well-established role of COP1 in degradation of positive regulators of photomorphogenesis in the dark (Lau and Deng 2012) is, therefore, extended to degradation of negative regulators in light (Zhu et al. 2015; Chen et al. 2015).

The positive side of COP1/SPA

COP1 and SPAs are commonly considered negative regulators of photomorphogenic development that enhance the degradation of positive regulators in the dark and negative

regulators in light (Lau and Deng 2012; Zhu et al. 2015; Chen et al. 2015). However, under certain conditions, the suppressive role of COP1 in regulation of photomorphogenesis is reversed (Cao et al. 2000; Boccalandro et al. 2004); if this is also true for SPAs, then this is not known. It has been shown that cotyledon unfolding in response to red light pulses and expression of several light-induced genes are reduced in weak *cop1* mutants compared with the wild type (Cao et al. 2000; Boccalandro et al. 2004). Moreover, overexpression of COP1 enhances cotyledon unfolding and reduces hypocotyl elongation in red light, whereas COP1 overexpression has the opposite effect in far-red and blue light (Boccalandro et al. 2004). Thus, the existence of a negative regulator of (red) light responses has been proposed that is negatively regulated by COP1 (Boccalandro et al. 2004). The B-box transcription factors SALT TOLERANCE (STO)/B-BOX DOMAIN PROTEIN 24 (BBX24) and SALT TOLERANCE HOMOLOGUE (STH)/BBX25 match this profile. They are targeted for degradation by COP1 and at least transiently stabilized in light but, in contrast to most COP1 targets, negatively regulate photomorphogenesis (Tab. 1) (Indorf et al. 2007; Gangappa et al. 2013). Thus, increased levels of STO/BBX24 and STH/BBX25 in *cop1* mutant background would repress photomorphogenesis and COP1 overexpression would remove this negative regulation. If turnover of STO/BBX24 and STH/BBX25 also depends on SPAs, then it has not been investigated. The effect of STO/BBX24 on hypocotyl growth is most prominent in red light, which is consistent with the observation that COP1-enhanced photomorphogenesis is restricted to red light (Indorf et al. 2007). However, no preference for red light has been found for STH/BBX25 (Gangappa et al. 2013) and experimental evidence that STO/BBX24 and/or STH/BBX25 are responsible or at least contribute to COP1-enhanced promotion of photomorphogenic responses in red light is lacking.

How to achieve wavelength specificity

Transcriptome analyses of light responsiveness at the seedling stage show that only UV-B light regulates a unique set of genes, while there is extensive overlap between genes regulated by red, far-red, blue, and white light (Peschke and Kretsch 2011). This may reflect that phytochrome and cryptochrome downstream signaling pathways converge on the COP1/SPA complex, whereas UVR8 uses a distinct pathway not depending on SPAs (Oravecz et al. 2006). It is important to note that the work by Peschke and Kretsch (2011) did not directly investigate UVR8-dependent gene regulation, and the use of monochromatic UV-B light in this study differs from other studies, where UV-B supplemented white light has been used (Tilbrook et al. 2013 and references therein). However, even in the visible wavelengths, a limited number of genes were found to respond to specific wavelengths in whole seedling transcriptome analyses, which leads to the question how such specificity could be achieved (Peschke and Kretsch 2011). It should also be noted that wavelength-specific responses in cell types with low representation in whole seedlings, such as meristematic tissues, may have been obscured in the whole seedling approach, and that seedlings may not be representative for other developmental stages. Thus, the number of genes regulated by specific wavelengths is likely higher than anticipated based on the transcriptome analyses with whole seedlings.

Signaling pathways working in parallel to the COP1/SPA complex certainly contribute to wavelength-specific light responses. For instance, specific red:far-red light ratios perceived by phyB regulate gene expression requiring PIF4, PIF5, and PIF7, while CRY2 could achieve blue light specific control of target genes dependent on members of the CRY2 INTERACTING BHLH (CIB) family (Liu et al. 2008a, 2013; Casson et al. 2009; Li et al. 2012; Hornitschek et al. 2012).

However, the COP1/SPA complex could also confer specificity through the specific properties and combinations of the different SPA proteins. The COP1/SPA complex is tetrameric and consists of two COP1 and either two identical or different SPA proteins, leading theoretically to ten different possible COP1/SPA complexes (Zhu et al. 2008). SPA proteins in *Arabidopsis* are only partially redundant and contribute to different developmental responses (Fig. 2) (Laubinger and Hoecker 2003; Laubinger et al. 2004; Fittinghoff et al. 2006; Rolauffs et al. 2012; Pacín et al. 2013). Furthermore, cryptochromes and phytochromes only interact with a subset of the SPA proteins (Suppl. Figure 1). If the combination of SPA proteins in the COP1/SPA complex defines the substrate specificity of the complex, binding of a specific cryptochrome or phytochrome to a specific combination of SPAs could result in wavelength-specific stabilization of a particular substrate. SPA1, for instance, is required for normal degradation of CO, HFR1, and HY5 (Yang et al. 2005a; Yang and Wang 2006; Laubinger et al. 2006), which may be due to specificity for these substrates being limited to only SPA1; alternatively, the reduced degradation of CO, HFR1, and HY5 in the *spa1* mutant might be due to total SPA levels that are too low in the absence of SPA1 rather than to a specific requirement for SPA1. Moreover, HY5 stabilization during de-etiolation is further enhanced in *spa1 spa2* than the *spa3 spa4* background, suggesting that SPA1 and SPA2 play a dominant role in regulation of HY5 turnover (Zhu et al. 2008). This finding is consistent with the predominant function of SPA1, SPA2, and HY5 during de-

etiolation and at the seedlings stage, whereas SPA3 and SPA4 are mainly required in adult plants, where the function of HY5 is less pronounced than in seedlings (Hardtke et al. 2000; Laubinger et al. 2004). However, if HY5 is mainly stabilized in *spa1 spa2* because it is a better target for COP1/SPA1/SPA2 than for COP1/SPA3/SPA4 complexes or if SPA1 and SPA2 are simply the most abundant SPAs during de-etiolation is still to be determined. Work on light-induced turnover of SPA1 and SPA2 has shown that light quality-specific regulation of specific subpopulations of COP1/SPA complexes occurs (Balcerowicz et al. 2011; Chen et al. 2015). Thus, it appears possible that plants achieve a wavelength-specific regulation of the turnover of different substrates by combining COP1 with specific sets of SPA proteins. The expression of the four *SPA* genes in *Arabidopsis* also depends on the developmental stage and the tissue (Zhu et al. 2008), suggesting that the competence to degrade different substrates varies with time and differs between different parts of the plant. Measuring and comparing the protein levels of the different SPAs under different light conditions and at different developmental stages and testing the degradation of different substrates in different *spa* mutants and transgenic lines that overexpress specific SPA dimers in the absence of all other SPAs could elucidate to what extent different SPA homo- and heterodimers confer substrate-specificity to different COP1/SPA complexes.

Evolutionary origin of SPAs

The split of the *SPA* gene lineage into *SPAs* similar to either *SPA1*/*SPA2* or *SPA3*/*SPA4* from *Arabidopsis* relies on a gene duplication event preceding the evolution of monocots and dicots; later, duplications in individual taxonomic entities further expanded the *SPA* gene diversity. (Ranjan et al. 2014). There are two *SPA* genes, *SPAa* and *SPAb*, in the moss *Physcomitrella patens*, which are highly similar and possibly derive from a recent duplication of an *SPA1*/*SPA2* ortholog (Ranjan et al. 2014). In contrast to flowering plants, where *COP1* typically is a single copy gene, the *COP1* gene family expanded in *Physcomitrella* and consists of nine members (Richardt et al. 2007; Ranjan et al. 2014). Expression of the *Physcomitrella* COP1 with the highest sequence similar to *Arabidopsis* COP1 largely complements the *Arabidopsis cop1-4* and *cop1-5* mutants (Ranjan et al. 2014). This suggests that COP1 provides a core function and is under negative selection, which precludes evolutionary divergence. Conversely, expression of SPAb from *Physcomitrella* did not complement (not even partially) the defects of *Arabidopsis spa1 spa2 spa3* and *spa1 spa3 spa4* mutants; rice SPA1 does not complement the light hypersensitivity of *spa1 spa3 spa4* plants, but at least partially inhibits de-etiolation of *spa1 spa2 spa3* seedlings in the dark (Ranjan et al. 2014). The failure of (full) complementation has been interpreted as an indication that SPA neo- and/or sub-functionalization occurred in the course of land plant evolution and that SPAs from one species, therefore, are functionally not sufficiently similar (e.g., regarding substrate-specificity or regulation by different photoreceptors) to SPAs from other species to allow substitution. Thus, COP1 might provide the core function of the COP1/SPA complex, which is modified by divergent SPA proteins that may place COP1 activity under the control of light (Ranjan et al. 2014). Potential substrates of the COP1/SPA complex, such as orthologs of *Arabidopsis* HY5 and CO, are present in *Physcomitrella* (Shimizu et al. 2004; Zobell et al. 2005; Yamawaki et al. 2011), and it will be interesting to determine if they are substrates of the *Physcomitrella* COP1/SPA complex—if COP1 and SPAs form a complex in *Physcomitrella* at all. Given that SPAs from *Physcomitrella* are not equivalent to *Arabidopsis* SPAs, it is also an open question if the activity of a potential COP1/SPA complex in *Physcomitrella* would be under the control of cryptochromes and phytochromes and—if yes—if the regulation would rely on similar mechanisms as in *Arabidopsis*.

Outlook and conclusions

Binding of phytochromes and cryptochromes to members of the SPA protein family may only be the first step in a cascade of molecular events that down-regulate the E3 ubiquitin ligase activity of the COP1/SPA complex in a time range from minutes to hours or even days. This includes COP1 nuclear exclusion, reduced COP1/SPA complex stability and reorganization, and SPA protein turnover. Such multilayer regulation may be the result of an evolutionary process leading to a robust and highly versatile control mechanism to allow rapid induction of gene expression in response to very low light intensities and short light pulses and at the same time inhibiting the activation of light signaling under inappropriate conditions, such as in dark-grown seedlings. Over the past few years, significant progress has been made toward understanding the role of SPAs; yet, this work also taught us what we still do not know and what questions we have to answer to fully understand how COP1 and SPA proteins link light perception with gene expression.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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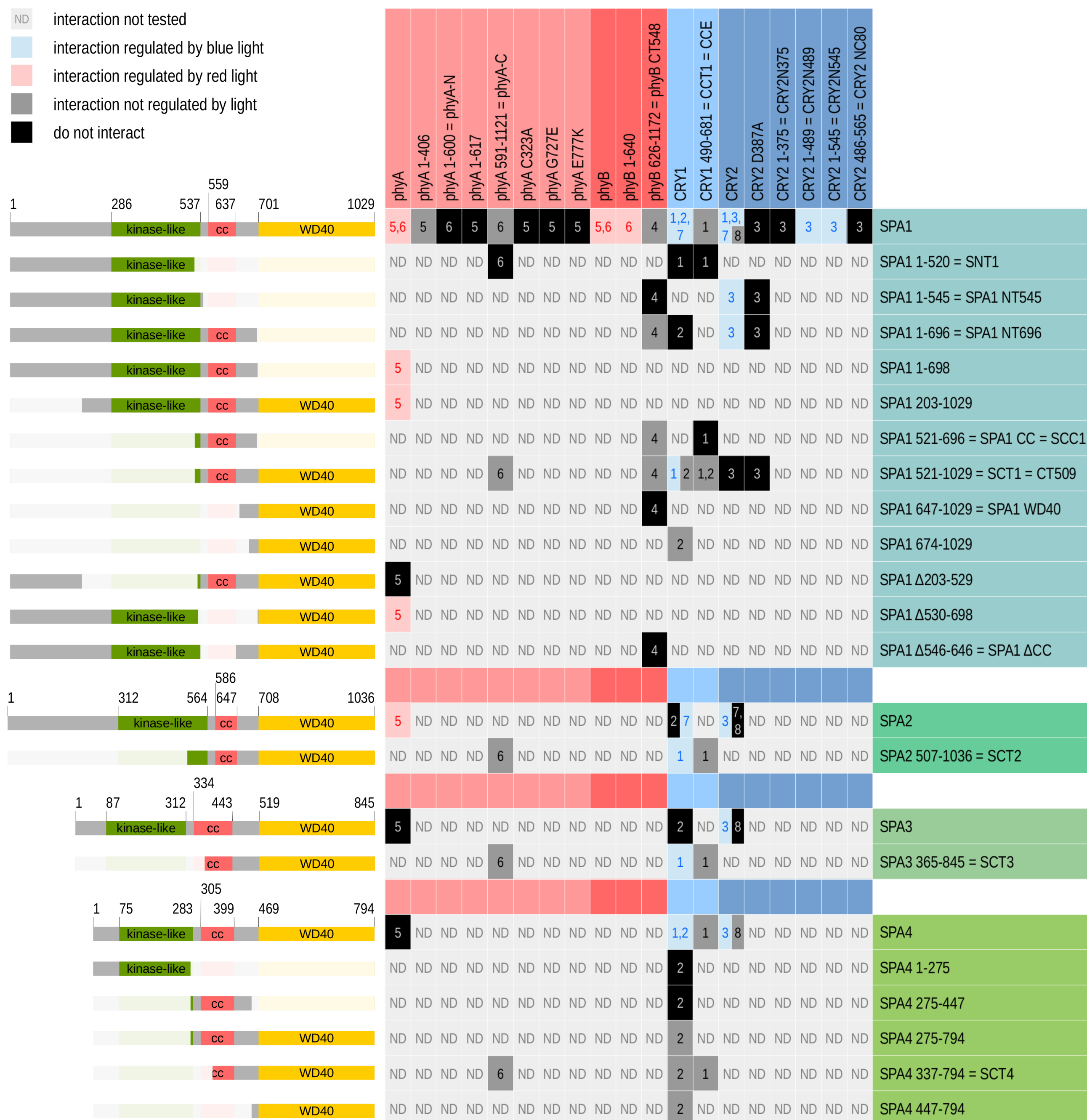
Supplemental Figures



Suppl. Fig. 1 Summary of phytochrome and cryptochrome fragments and mutants interacting with different domains of SPA proteins

Schematic drawing of the respective SPA proteins (full-length or fragments) are shown on the left. Numbers indicate amino acid positions. Commonly used abbreviations for the different SPA, phytochrome and cryptochrome constructs are shown on the right side and on the top, respectively. Black, no interaction, dark grey, light-independent interaction; red or blue, interaction requires red or blue light or is at least enhanced by light; ND, interaction has not been tested. Numbers 1 to 8

give the references: 1 = (Lian et al. 2011); 2 = (Liu et al. 2011); 3 = (Zuo et al. 2011); 4 = (Zheng et al. 2013); 5 = (Sheerin et al. 2015); 6 = (Lu et al. 2015); 7 = (Chen et al. 2015); 8 = (Weidler et al. 2012).

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Phytochrome A Signal Transduction in the Absence of FHY1 and FHL

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Abbreviations: Col, *Arabidopsis thaliana* ecotype Columbia; *cop1,* CONSTITUTIVE PHOTOMORPHOGENIC 1 phenotype; FHL, FHY1-like; FHY1, FAR-RED ELONGATED HYPOCOTYL 1; FR, far-red light; GFP/YFP/CFP, green/yellow/cyan fluorescent protein; HIR, high irradiance response; LFR, low fluence response; NLS, nuclear localization signal; NES nuclear export signal; Pfr/PfrA, FR absorbing form of phytochromes/phyA; phyA-E, phytochrome A-E; Pr/PrA, R absorbing form of phytochromes/phyA; R, red light; TF, transcription factor; VLFR, very low fluence response.

Abstract

The R/FR photoreceptors of the phytochrome family control a multitude of responses in plants. PhyA is essential for far-red light perception, which is of particular importance for germination and seedling establishment in canopy shade. The light-mediated nuclear translocation of phyA is one of the key steps in FR signalling and requires the interaction with the two functional homologs FHY1 and FHL. Additionally, FHY1/FHL bind to phyA downstream signalling components such as HFR1 and LAF1 suggesting their involvement in mediating the assembly of phyA signalling complexes in the nucleus. Here, we show that an artificial FHY1, consisting of an SV40 NLS and the phyA binding site of FHY1 and a YFP tag as spacer in between complements the *fhy1 fhl* double mutant, despite the lack of the HFR1 and LAF1 interaction domain. Moreover, phyA fused to an NLS is constitutively nuclear localized and active also in absence of FHY1 and FHL, suggesting that FHY1 and FHL are not required for phyA downstream signalling in the nucleus. However, we found that lines expressing phyA-NLS are strongly hypersensitive to R and that strong expression of constitutively nuclear localized phyA results in a *cop* phenotype. Thus, FHY1/FHL mediated phyA nuclear transport might be important to avoid photomorphogenesis in the dark and to restrict the activity of phyA to the FR light range of the light spectrum.

Introduction

Light plays an important role throughout the life of plants, which do not only use light as a source of energy but also as a source of information. By measuring the direction, the intensity, the spectral composition and temporal patterns of incident light, plants gain important information about their environment. As sessile organisms, plants rely on such information to adapt growth and development to the environmental conditions. For light perception, plants own different classes of photoreceptors. These include the blue light /UV-A sensing cryptochromes (CRY), phototropins and ZEITLUPE/FLAVIN-BINDING, KELCH REPEAT, F BOX 1/LOV KELCH PROTEIN 2 family proteins (ZTL/FKF1/LKP2), the UV-B receptor UVB-RESISTANCE 8 (UVR8) and the phytochromes, which mainly work in the red (R) and far-red (FR) range of the light spectrum (Galvao and Fankhauser, 2015). Phytochromes contain a linear tetrapyrol as chromophore, which is bound to a conserved cysteine residue. They can exist in two different forms, the inactive Pr form and the biologically active Pfr form, which have absorption peaks in R and FR, respectively. Phytochromes are synthesised in Pr and can reversibly interconvert between Pr and Pfr by absorption of light (Rockwell et al., 2006; Burgie and Vierstra, 2014). In Arabidopsis, the phytochrome family includes five members classified as phytochrome A to E (phyA-E) (Xu et al., 2015). Among them phyA and phyB are most prominent and mediate a broad range of responses, whereas phyC-E are important under very specific conditions. Furthermore, depending on their stability in light phytochromes are divided into two groups, the light labile (type I) and the light stable (type II) phytochromes (Furuya, 1993). PhyB-E are type II phytochromes; phyB is the most important phytochrome in light grown and adult plants. To initiate downstream signalling, phyB requires a high Pfr:Ptot (Ptot=Pr+Pfr) ratio, as present in R or white light (W). In contrast, phyA is the only type I phytochrome present in Arabidopsis. It triggers signal transduction in response to very low Pfr:Ptot ratios typically established by irradiation with FR or weak light of any wavelength. Thus, phyA has a dual function, working as a receptor for weak light in the VLFR (very low fluence response) mode and as a sensor for FR in the HIR (high irradiance response) mode (Casal, 1998). Both response modes are of ecological relevance and it has been shown that phyA is essential for germination and seedling establishment in FR-rich environments, such as in the understory of forests (Yanovsky et al., 1995; Botto et al., 1996; Nagy and Schafer,

2002; Casal et al., 2014).

Phytochromes localize to the cytosol in the dark and translocate into the nucleus upon activation by light. PhyA and phyB use distinct molecular mechanisms for their nuclear translocation. PhyA nuclear import depends on the functional homologs FHY1 (FR ELONGATED HYPOCOTOYL 1) and FHL (FHY1-LIKE) (Hiltbrunner et al., 2005; Hiltbrunner et al., 2006; Rosler et al., 2007; Genoud et al., 2008). FHY1 and FHL are small plant-specific proteins containing a NLS (nuclear localization signal) and a putative NES (nuclear export signal) motif at the N-terminus, and a phyA binding site at the very C-terminus. Both the NLS and the phyA binding motif are essential for proper FHY1/FHL function, whereas the NES seems to be dispensable (Zeidler et al., 2004; Zhou et al., 2005; Hiltbrunner et al., 2006; Toledo-Ortiz et al., 2010). Consistent with their functional relevance, the NLS and the phyA binding site are highly conserved among FHY1/FHL homologs from monocots and dicots and we found that an artificial FHY1 consisting of an SV40 NLS and the phyA binding site linked by YFP as a spacer can substitute for endogenous FHY1 (Genoud et al., 2008).

Transcription factors (TFs) such as LAF1 (LONG AFTER FAR-RED LIGHT 1 (Ballesteros et al., 2001), HFR1 (LONG HYPOCOTYL IN FAR-RED 1) (Ni et al., 1998; Fankhauser and Chory, 2000; Soh et al., 2000), HY5 (LONG HYPOCOTYL 5) (Oyama et al., 1997) and PIF3 (PHYTOCHROME INTERACTING FACTOR 3) (Tepperman et al., 2006) are signaling components downstream of phyA involved in the regulation of phyA-responsive genes. It has been shown that LAF1 and HFR1 interact with FHY1 and FHL (Yang et al., 2009). Moreover, also PIF3 and HY5 may bind to FHY1, though the interaction between PIF3 and FHY1 might be indirect (Chen et al., 2012). It is important to note that the interaction of FHY1 and HY5 could not be confirmed in an independent study; thus, it is unclear whether or not HY5 binds to FHY1 (Jang et al., 2013). Since FHY1 and FHL interact with several transcription factors, it has been proposed that they play a role in the assembly of phyA signalling complexes in the nucleus besides mediating phyA nuclear transport.

Previously, we have shown, that constitutively nuclear localized phyA is physiologically active and restores the sensitivity to FR in the absence of FHY1 (Genoud et al., 2008). Thus, FHY1 seem not to be essential for FR signalling in plants expressing a phyA version that enters the nucleus in an FHY1/FHL independent manner. However, the transcript level

of *FHL* is upregulated in *fhy1* mutant background, and it has been suggested that FHL is sufficient for the assembly of phyA signalling complexes in the absence of FHY1 (Yang et al., 2009). Therefore, while it is well-established that FHY1 and FHL are essential for phyA nuclear transport, it is still unclear whether or not they are required for phyA downstream signalling after phyA has been transported in to the nucleus. In order to answer this question we analysed the activity of constitutively nuclear localized phyA in *phyA fhy1 fhl* triple mutant background. Moreover, we also tested the activity of artificial FHY1 in the absence of FHY1 and FHL.

Results

An artificial FHY1 is functional *in vivo*

We have previously shown that an artificial FHY1 consisting of an SV40 NLS and the phyA interaction domain of Arabidopsis FHY1 (FHY1 167-202) separated by YFP as spacer is functional (Genoud et al., 2008). 35S promoter driven expression of this artificial FHY1 in *fhy1-1* mutant background resulted in strong hypersensitivity to FR, similar to seedlings overexpressing wild-type FHY1 (Desnos et al., 2001; Zeidler et al., 2004; Genoud et al., 2008). In contrast, expression of p35S:YFP-NLS-FHY1 167-202 (i.e. a construct containing the YFP at the N-terminus instead of in between the NLS and FHY1 167-202) was almost completely inactive when expressed in *fhy1-3 fhl-1* double mutant background. Thus, it has been proposed that artificial FHY1 is not fully active and that the spacer region of FHY1, which is replaced by YFP in artificial FHY1, might be essential for the interaction with other proteins. Indeed, FHY1 and FHL lacking the C-terminal phyA binding site were shown to bind HFR1 and LAF1 and therefore it has been concluded that FHY1 and FHL are required to assemble phyA/transcription factor complexes for downstream signalling (Yang et al., 2009).

In our previous study we expressed artificial FHY1 in *fhy1-1* background, where FHL is still present; therefore it is possible that artificial FHY1 could compensate for the reduced nuclear transport activity in *fhy1-1*, while FHL, for which the transcript levels are threefold higher in *fhy1-3* than the wild-type (Yang et al., 2009), was sufficient for the assembly of

HFR1/LAF1/phyA signalling complexes in the nucleus. This would explain why p35S:SV40 NLS-YFP-FHY1 167-202 complements *fhy1-1*, while p35:YFP-SV40 NLS-FHY1 167-202 is only very weakly active in *fhy1-3 fhl-1* (Genoud et al., 2008; Yang et al., 2009). However, it should be noted that the position of the constructs used in the two studies are not identical; Yang et al. (2009) used a construct containing the YFP at the N-terminus, whereas we used YFP to substitute for the spacer present in natural FHY1, which separates the NLS and the phyA binding site.

To clarify whether the position of YFP might be responsible for the different results in the two studies rather than the presence of wild-type FHL we several independent transgenic lines expressing p35S:SV40 NLS-YFP-FHY 167-202 in *fhy1-3 fhl-1* double mutant background. We tested the functionality of this artificial FHY1 in two typical FR-HIRs: the inhibition of hypocotyl elongation and the accumulation of anthocyanin (Fankhauser and Casal, 2004; Bae and Choi, 2008; Li et al., 2011). *fhy1-3 fhl-1* lines expressing the p35S:NLS-YFP-FHY1 167-202 showed strong inhibition of hypocotyl growth in FR similar to the wild-type and *fhy1-3* seedlings expressing p35S:YFP-FHY1 (Figure 1A). Furthermore, also in terms of anthocyanin accumulation the four independent *fhy1-3 fhl-1* p35S:NLS-YFP-FHY1 167-202 lines displayed a FR-HIR phenotype similar to wild-type (Figure 1B), suggesting that NLS-YFP-FHY1 167-202 is fully functional in the absence of endogenous *FHY1* and *FHL*. FHY1 and FHL have been shown to form phyA dependent nuclear bodies upon light exposure (Hiltbrunner et al., 2005; Hiltbrunner et al., 2006). Similar, to FHY1 and FHL, also artificial FHY1 formed nuclear bodies *fhy1-3 fhl-1* seedlings exposed to light (Figure 1C).

We therefore come to the conclusion that the fusion protein consisting of an SV40 NLS, YFP, and the phyA binding-motif of FHY1 is functional and behaves like native Arabidopsis FHY1. Given that artificial FHY1 lacks the amino acid region required for binding of HFR1 and LAF1 it appears unlikely that FHY1 and FHL are essential for the assembly of HFR1/LAF1/phyA signalling complexes and phyA downstream signalling, though we cannot exclude that they could enhance the formation or the stability of such complexes.

PhyA signalling in absence of FHY1 and FHL

Artificial FHY1 still contains the C-terminal 36 amino acid residues of wild-type FHY1, which are required for phyA binding. Though there is no evidence that any protein except for phyA binds to this motif, we cannot rule out the possibility that unknown factors required for phyA downstream signalling might bind to the phyA binding motif. Therefore, in a hypothetical scenario artificial FHY1 could play a role in HFR1/LAF1-independent phyA downstream signalling. To investigate this possibilty, we tested the phenotypic consequence of expressing a constitutively nuclear localized version of phyA under the control of the endogenous phyA promoter (pPHYA:PHYA-NLS-YFP) in a *phyA-211 fhy1-3 fhl-1* triple mutant. For this purpose we generated two independent lines (#10772 and #9494) by crossing *phyA-211* pPHYA:PHYA-NLS-YFP (#7129) and *fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP (#6900), respectively, into *phyA-211 fhy1-3 fhl-1* and thereafter selecting siblings in the F2 generation that were homozygous for *phyA-211 fhy1-3 fhl-1* and the transgene.

All pPHYA:PHYA-NLS-YFP expressing lines restored inhibition of hypocotyl growth and accumulation of anthocyanin in FR in *phyA-211* single-, *fhy1-3 fhl-1* double-, and *phyA-211 fhy1-3 fhl-1* triple-mutant background very efficiently (Figures 2A and B). Moreover, microscopic analysis showed that, as expected, phyA-YFP is unable to translocate into the nucleus in the absence of FHY1 and FHL, while fusing a NLS to phyA renders phyA nuclear accumulation light- and FHY1/FHL-independent. Moreover, phyA-NLS-YFP formed nuclear bodies similar to the control line expressing pPHYA:PHYA-YFP in *phyA-211* mutant background, demonstrating that recruitment of phyA into nuclear bodies is independent of FHY1 and FHL (Figure 2C).

We also crossed *fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP into *fhy1-3 fhl-1 phyB-9* and selected in the F2 generation for plants that are homozygous for the transgene an the mutant background, which we then crossed into *phyA-211 fhy-3 fhl-1*. F2 seedlings being homozygous for the pPHYA:PHYA-NLS-YFP transgene and *phyA-211*, *phyB-9*, *fhy1-3*, and *fhl-1* had strongly reduced hypocotyl growth in FR compared to D and accumulated high levels of anthocyanin (Figure 2E and F). Thus, as expected, FR responses of seedlings expressing phyA-NLS-YFP does not depend on phyB.

The phyA Y242H mutant allele contains a Y-to-H amino acid substitution at position 242 and has been shown to be constitutively in the active Pfr form. Thus, it binds to FHY1/FHL and the phyA downstream signalling factors PIF1 and PIF3 in a light-independent fashion (Su and Lagarias, 2007; Rausenberger et al., 2011). Constitutive binding of phyA Y242H possibly traps FHY1/FHL and inhibits efficient recycling of FHY1/FHL from the nucleus into the cytosol, which interferes with efficient phyA nuclear transport. However, fusing a NLS to phyA Y242H-YFP overcomes this defect and expression in wild-type background results in a strong *cop* phenotype (Rausenberger et al., 2011). Here, we transformed the pPHYA:PHYA Y242H-NLS-YFP construct into *fhy1-3 fhl-1* mutant background and isolated a homozygous line in the T2 generation; moreover, we also crossed this line into the p*hyA-211 fhy1-3 fhl-1* triple mutant and selected in the F2 generation for siblings being homozygous for the transgenes as well as for *phyA-211*, *fhy1-3*, and *fhl-1*. phyA Y242H-NLS-YFP was highly active in *fhy1-3 fhl-1* double- and *phyA-211 fhy1-3 fhl-1* triple-mutant background (Figures 2A and B). Phenotypically, dark-grown *fhy1-3 fhl-1* and *phyA-211 fhy1-3 fhl-1* seedlings expressing phyA Y242H-NLS-YFP exhibited a strong *cop* phenotype and in FR-light they displayed an inhibition of hypocotyl growth similar to wild-type (Figure 2D). This data further confirm that FHY1 and FHL are not required for phyA downstream signalling in the nucleus.

PhyA-NLS still relies on phyA downstream signalling components for induction of FR responses

In order to analyze if fusing a NLS to phyA specifically overcomes defects in nuclear transport, but not in downstream signaling, we investigated the phenotypic effect of the expression of a constitutively nuclear localized phyA in mutant seedlings deficient for HFR1 and HY5. To this end we crossed the *phyA-211* pPHYA:PHYA-NLS-YFP (#7129) transgenic line into *hfr1-4* and *hy5-215*, respectively, and selected in the F2 generation of plants homozygous for the transgene, *phyA-211* and either *hfr1-4* or *hy5-215*.

When grown in continuous FR light of different fluence rates, wild-type seedlings showed a stronger inhibition of hypocotyl growth than the *hy5-215* and *hfr1-4* mutants. However, both these mutants had much shorter hypocotyls than the *fhy1-3 fhl-1* mutant, in which

inhibition of hypocotyl growth is completely insensitive to FR. Interestingly, while expression of pPHY1:PHYA-NLS-YFP fully complements the strong *fhy1-3 fhl-1* double mutant it only slightly improves the response to FR in *hy5-215* and *hfr1-4* (Figure 3A). The residual effect of phyA-NLS-YFP on hypocotyl growth in *hy5-215* and *hfr1-4* is possibly due to increased activation of HFR1- and HY5-independent signalling pathways. Thus, constitutive nuclear localized phyA still requires HFR1 and HY5 for downstream signalling, while FHY1 and FHL become dispensable in presence of phyA-NLS-YFP (Figure 3A). Moreover, in terms of anthocyanin accumulation it is even more evident that expression of pPHYA:PHYA-NLS-YFP in the *phyA-211 hy5-215* double mutant background is unable to restore the wild-type phenotype (Figure 3B). In contrast to *hy5-215*, the *hfr1-4* mutant has only a very weak phenotype regarding anthocyanin accumulation in FR, which has been observed previously. However, phyA-NLS-YFP caused strongly increased anthocyanin levels in *phyA-211* but not in *phyA-211 hfr1-4.* These data largely confirm that adding a NLS to phyA only circumvents the need of FHY1/FHL for nuclear transport but not the requirement of downstream signalling factors, such HY5 and HFR1. Additionally, recruitment of phyA-NLS-YFP into light-induced nuclear bodies is not affected in absence of either HFR1 or HY5 (Figure 3C).

Plants expressing phyA-NLS-YFP are strongly hypersensitive to R

PhyB is the primary receptor for R, but it is well-known that phyA also contributes to R responses and even plays a dominant role in early R signalling (Tepperman et al., 2006). Thus, we measured fluence rate response curves for the wild-type and *phyA-211* expressing either pPHYA:PHYA-YFP (#8210) or pPHYA:PHYA-NLS-YFP (#7129). Both in R and FR the pPHYA:PHYA-YFP expressing line was similar to the wild-type control, while expression of pPHYA:PHYA-NLS-YFP resulted in hypersensitivity to R and FR over the full range of fluence rates (Figure 4C). It is interesting that pPHYA:PHYA-NLS-YFP expressing plants are about 10-fold more hypersensitive to R than to FR, suggesting that the action spectrum of this line is different from that of the wild-type. FHY1 and FHL would therefore not only be required for nuclear transport of phyA but also for shaping the phyA action action spectrum, which has been predicted by mathematical models (Rausenberger et al., 2011).

High expression levels of phyA-NLS-YFP result in a *cop* phenotype

Compared to the wild-type dark-grown *phyA-211* pPHYA:PHYA-NLS-YFP seedlings (line #7129) have slightly unfolded cotyledons and reduced apical hook formation, which is consistent with previous work (Toledo-Ortiz et al., 2010). However, we found that many *phyA-211* pPHYA:PHYA-NLS-YFP lines that we isolated, such #7137 and #7146, had a clear *cop* phenotype when grown in D with fully opened cotyledons and reduced hypocotyl growth. We used different light treatments to induce germination, including 3 h R, 3 h R followed by a long-wavelength FR (RG9) pulse, or 5 min R followed by 2 h 55 min D and an RG9 pulse ("true-dark" (Leivar et al., 2008), but even under "true-dark" conditions lines #7137 and #7146 had a clear *cop* phenotype (Figure 5). Using a GFP-specific antidoby for immunoblot analysis we compared the phyA-NLS-YFP levels in lines #7129, #7137, and #7146. Consistent with the strong *cop* phenotype line #7137 has the highest expression levels, while phyA-NLS-YFP levels are only slightly higher in #7146 than in #7129, which, however, appears to be sufficient to induce de-etiolation in D (Figure 5). We conclude that high levels of phyA in the nucleus can induce downstream signalling in the absence of light, i.e. even if phyA is in the inactive Pr form.

Discussion

It has been widely accepted that FHY1 and its homolog FHL regulate a critical step in the FR signaling pathway by controlling the nuclear import of phyA (Hiltbrunner et al., 2006; Rosler et al., 2007; Genoud et al., 2008). However it has been controversially discussed if FHY1 and FHL are also required for phyA downstream signalling in the nucleus. Here, we addressed this question in detail and provide conclusive evidence that FHY1 and FHL are essential for nuclear transport of phyA but not for downstream signalling of nuclear localized phyA.

Several transcription factors involved in phyA downstream signalling interact with FHY1 and/or FHL. Both HFR1 and LAF1 bind to FHY1 and FHL independently of the phyA binding site (Yang et al., 2009). Also HY5 and PIF3 have been shown to interact with FHY1, though binding of HY5 to FHY1 could not be confirmed in an independent study (Chen et al., 2012; Jang et al., 2013). Moreover, PIF3 binding might be indirect through, for instance, phyA. Thus, it has been proposed that FHY1 and FHL are essential for the assembly of phyA signalling complexes. In contradiction to this model, we have previously shown that expression of phyA-NLS fully complements the *fhy1* mutant. Furthermore, an artificial FHY1 consisting of an SV40 NLS fused to YFP and the C-terminal phyA binding site of FHY1 is active *in planta* and restores FR responses when expressed in *fhy1* (Genoud et al., 2008). It is important to note that this artificial FHY1 lacks the binding sites for HFR1 and LAF1, while it may or may not contain the sites required for interaction with HY5 and PIF3. These data imply that FHY1 is not required for responses to FR in seedlings expressing phyA-NLS or artificial FHY1. However, FHL transcript levels are threefold upregulated in the absence of FHY1 (Yang et al., 2009). If also FHL protein levels are increased in *fhy1-3*, FHL may compensate for the lack of FHY1 in lines expressing phyA-NLS or artificial FHY1 in *fhy1* mutant background. Thus, it has not been possible to decide if FHY1 and FHL are required for phyA downstream signalling in the nucleus or if they only mediate phyA nuclear transport. Here, we expressed phyA-NLS-YFP and artificial FHY1 in *fhy1 fhl* mutant background. Artificial FHY1 and constitutively nuclear localized phyA both complement the *fhy1 fhl* double mutant regarding hypocotyl growth inhibition and accumulation of anthocyanin in FR. Thus, we conclude that FHY1 and FHL are not essential for downstream signalling of nuclear localized phyA, while they are

required for phyA nuclear accumulation in light. FHY1 and FHL may or may not be involved in the assembly of phyA signalling complexes, which may or may not modulate phyA downstream signalling. However, here we demonstrate that these FHY1/FHL-dependent signalling complexes are not required for hypocotyl growth inhibition and anthocyanin accumulation; moreover, this might also be true for other FR responses though experimental evidence still lacking.

Artificial FHY1 consists of YFP containing a SV40 NLS and the phyA binding site of FHY1 (FHY1 167-202) fused the N- and C-terminus, respectively. In artificial YFP simulates the "spacer" in natural FHY1. This spacer region is roughly 150 to 250 amino acid residues in length without any annotated functional motifs and with very low sequence similarity between FHY1/FHL proteins from different species. However, it is interesting that this spacer region is present in all FHY1/FHL proteins and, though variable in length, rarely shorter than 150 amino acid residues. Thus, it might be that despite the lack of similarity at the level of the primary sequence the 3D structure is similar. Yet, we have shown that YFP can substitute for the spacer, suggesting that this explanation is unlikely to be true. Alternatively, the spacer might simply provide sufficient flexibility, which might be important for simultaneous binding of phyA to the C-terminal phyA binding site and importin alpha and other components of the nuclear transport machinery to the NLS. In this regard it is interesting that Yang et al. (2009) have shown that YFP-NLS-FHY1 167-202 is only very weakly active and does not complement the *fhy1 fhl* double mutant. Our interpretation is that fusing the NLS directly to the phyA binding site and adding the YFP at the very N-terminus of the construct (instead of inserting YFP as a spacer between the NLS and the phyA binding site) does not allow phyA and importin alpha to bind simultaneously, which would be possible in natural FHY1/FHL and our artificial FHY1, and which is required for phyA nuclear transport. Based on the Pr and Pfr absorption spectra, phytochromes are expected to have an action peak in R. While phyB indeed is most active in R, phyA responses have an action peak in FR. Using a mathematical modelling approach we previously explored potential molecular mechanisms involved in shifting the phyA action peak from R to FR. FHY1/FHL-dependent nuclear transport was identified as one potential mechanism (also referrred to as "shifting module") but also the existence of FHY1/FHL-independent shifting modules working in parallel was predicted. Interestingly, lack of only one shifting module was predicted not to shift the phyA action peak but to result in a broadening of the peak so that it extends into the R range of the light spectrum. Adding a

NLS directly to phyA renders phyA nuclear transport independent of FHY1 and FHL and therefore abolishes one shifting module. Here, we have shown that seedlings expressing phyA-NLS still show a stronger response to FR than to R but that the relative increase of the response to R is about 10-fold higher than to FR. Thus, phyA-NLS expressing seedlings still have a phyA action peak in FR but the peak is obviously broader than in the wild-type and extends into the R region of the spectrum; this result is in agreement with the prediction of the mathematical modelling approach. In conclusion, FHY1/FHL-mediated nuclear transport of phyA contributes to restricting the phyA activity to FR, which, together with a recently described mechanism that confines the activity of phyB to wavelengths below 690 nm, enables plants to reliably distinguish between FR and R.

Dark-grown seedlings rapidly elongate and have folded cotyledons and an apical hook that protects the apical meristem from mechanical damage. This developmental programme, skotomorphogenesis, allows seedlings germinating from seeds in the soil to reach the surface, where light perceived by phytochromes and cryptochromes induces photomorphogenesis. This programme is suppressed in constitutively photomorphogenic mutants, which therefore have a lower probability than the wild-type of successful establishment after germination in the soil. Thus, it can be assumed that under natural conditions there is strong selection against mutants that are constitutively photomorphogenic. We have shown that moderate expression of phyA-NLS-YFP reinstates FR induced anthocyanin accumulation and inhibition of hypocotyl growth in *phyA* and *fhy1 fhl* but that only slightly increased levels result in a constitutively photomorphogenic phenotype. In contrast, we have never observed such a *cop* phenotype for plants expressing different levels of wild-type phyA (i.e. of phyA that relies on FHY1/FHL for nuclear transport). Weak but significant phyA-dependent induction of gene expression in *fhy1 fhl* background suggests that even in the absence of FHY1 and FHL residual amounts of phyA are present in nuclei of dark-grown seedlings (Kami et al., 2012; Pfeiffer et al., 2012); however, the amount of phyA in nuclei of dark-grown plants expressing phyA-NLS is several orders of magnitudes higher. We suggest that the activity of phyA-NLS-YFP in D is likely due to weak interaction of the inactive Pr form with downstream signalling factors, such as PIF1, PIF3, SPA1, or SPA2, which results in constitutive photomorphogenesis. Thus, in the hypothetical scenario that endogenous phyA would contain a NLS it might be very difficult for plants to regulate the levels of phyA sufficiently precise to ensure proper photomorphogenesis in light without suppressing

skotomorphogenesis in the dark. In contrast, FHY1 and FHL provide a highly robust mechanism that reliably prevents phyA from accumulating in the nucleus in the dark, while efficiently transporting it into the nucleus in light. It is interesting that nuclear transport of phyB is much less tightly regulated than nuclear transport of phyA (Klose et al., 2014). The reason for this might be that there is simply no need for a more tight control of phyB nuclear transport. Induction of light signalling by phyB is much less sensitive than by phyA and therefore even high levels of phyB Pr in the nucleus would not be sufficient to induce photomorphogenesis in dark-grown seedlings (Huq et al., 2003; Matsushita et al., 2003). Thus, there is no selection pressure that would drive the evolution of a phyB nuclear transport mechanism that is as selective as FHY1/FHL-dependent phyA nuclear transport.

In summary, here we have shown that FHY1 and FHL are required for phyA nuclear transport but that phyA, once it is in the nucleus, does not require FHY1 and FHL for downstream signalling. In addition, our data suggest that FHY1 and FHL play a role in shaping the phyA action spectrum by reducing the phyA activity in the R range of the light spectrum. Finally, FHY1/FHL provide a highly robust nuclear transport system for phyA, which prevents phyA from accumulating in the nucleus and inducing light signalling in the dark.

Experimental procedure

Constructs, transgenic plants

Constructs coding for pPHYA:PHYA-NLS and pPHYA:PHY Y242H-NLS have been described (Rausenberger et al., 2011).

pPPO72-FHY1 167-202 is a T-DNA vector containing a p35S:SV40 NLS-YFP-FHY1 167-202:terRbcS cassette and PPO as selectable marker. It was obtained as follows. The PPO selection marker cassette was cut from pWCO35 (Hanin et al., 2001) using PvuII/PstI and ligated into the SbfI/PmlI site of pCHF72-FHY1 167-202 (Genoud et al., 2008) to replace the BASTA selectable marker.

The plant expression vector pPPO30v1HA (encoding p35S:BamHI-XbaI-YFP-HA:terRbcS and containing PPO as selection marker) was generated by first cutting pCHF5 (Hiltbrunner et al., 2005) with PmeI/NcoI, and ligating in a StuI/NcoI fragment from pYES2 (Invitrogen) to generate pCHF5v1. pWCO35 (Rausenberger et al., 2011) was then cut with PvuII/PstI and this fragment ligated into PmlI/SbfI cut pCHF5v1 to generate pPPO5v1. Finally, EYFP was amplified by PCR from pPPO30 (Rausenberger et al., 2011) using the primers 5′-CGC GGA TCC CGC TCT AGA ATG GTG AGC AAG GGC GAG G-3′ and 5′-GTA CGT CGT ATG GGT AGC TAG CCT TGT ACA GCT CGT CCA TG-3′; the EYFP PCR fragment was used as template for another PCR using the primers 5′-CGC GGA TCC CGC TCT AGA ATG GTG AGC AAG GGC GAG G-3′ and 5′-GGA CTA GTT TAA GCG TAA TCT GGT ACG TCG TAT GGG TAG C-3′. The resulting PCR fragment was then cut with BamHI/SpeI and cloned into BamHI/XbaI cut pPPO5v1 to generate pPPO30v1HA. To obtain pPPO30v1HA-HFR1 (coding for p35S:HFR1-YFP-HA:terRbcS) we cut HFR1 from pBS II KS-HFR1 (Sheerin et al., 2015) using BamHI/SpeI and ligated it into the BamHI/XbaI site of pPPO30v1HA.

pPPO70-FHY1 is a T-DNA vector containing a p35S:YFP-FHY1:terRbcS cassette and PPO as selectable marker. It was obtained as follows. The PPO selection marker cassette was cut from pWCO35 (Hanin et al., 2001) using PvuII/PstI and ligated into the SbfI/PmlI site of pCHF70-FHY1 (Rausenberger et al., 2011) to replace the BASTA selectable marker.

Plant Material

Columbia (Col-0) ecotype of *A.thaliana* has been used in this report as wild-type. The *phyA-211, fhy1-3 fhl-1, fhy1-3, fhl-1, hfr1-4 and hy5-215* have been described previously (Reed et al., 1994; Oyama et al., 1997; Zeidler et al., 2001; Sessa et al., 2005; Zhou et al., 2005; Rosler et al., 2007). The transgenic lines *fhy1-3* p35S:YFP-FHY1*, fhy1-3 fhl-1* p35S:NLS-YFP-FHY1 167-202 (four independent lines: #9526, #9537, #9742, #9717), *phyA-211* pPHYA:PHYA-NLS-YFP*, fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP*, phyA-211* pPHYA-PHYA-YFP*, fhy1-3 fhl-1* pPHYA:PHYA Y242H-NLS-YFP*, fhy1-3 fhl-1* pPHYA:PHYA-YFP, and *hfr1-4* p35S:HFR1-YFP-HA were obtained by *Agrobacterium tumefaciens* (C58 strain)-mediated transformation using the floral dip method (Davis et al., 2009).

The *phyA-211 fhy1-3 fhl-1* line was obtained by crossing *phyA-211* into *fhl-1* and subsequently *phyA-211 fhl-1* into *fhy1-3 fhl-1* background. *phyA-211 fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP has been generated by crossing *phyA-211* pPHYA:PHYA-NLS-YFP*,* as in case of #10772, or, for #9494, *fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP into *phyA-211 fhy1-3 fhl-1. phyA-211 fhy1-3 fhl-1* pPHYA:PHYAY242H-NLS-YFP was obtained from *fhy1-3 fhl-1* pPHYA:PHYAY242H-NLS-YFP crossed into *phyA-211 fhy1-3 fhl-1; phyA-211 fhy1-3 fhl-1* pPHYA:PHYA-YFP *was* obtained by genetic crossing *phyA-211 fhy1-3 fhl-1* and *fhy1-3 fhl-1* pPHYA:PHYA-YFP. By crossing *hfr1-4* or *hy5-215* into *phyA-211* pPHYA:PHYA-NLS-YFP we obtained *phyA-211 hfr1-4* pPHYA:PHYA-NLS-YFP and *phyA-211 hy5-215* pPHYA:PHYA-NLS-YFP respectively. *phyA-211 phyB-9 fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP was obtained as follows. We crossed *phyB-9* into *fhl-1* and subsequently *phyB-9 fhl-1* into *fhy1-3 fhl-1*, resulting *phyB-9 fhy1-3 fhl-1*. *phyB-9 fhy1-3 fhl-1* was then crossed into *fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP to obtain *phyB-9 fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP. Finally we crossed *phyB-9 fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP into *phyA-211 fhy1-3 fhl-1* resulting in *phyA-211 phyB-9 fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP.

Seed sterilization and plating

Before analysis of hypocotyl length and anthocyanin accumulation, the seeds have been surface sterilized by shaking in 1 ml 70% ethanol with 0.05% Triton X-100 for 10 min

followed by 5 min incubation in 100% ethanol. After that they have been transferred on sterile filter paper in a hood, dried for approximately 30 minutes and spread onto Petri dishes containing half-strength Murashige and Skoog salts (Duchefa) and 1% phytoagar (Duchefa). Differently, for the measurements of the anthocyanin accumulation sterilized seeds have been plated on half-strength MS, 1% phytoagar supplemented with 1,5% sucrose.

Hypocotyl length, anthocyanin accumulation

Germination of Arabidopsis seeds has been induced by stratification for 4 d at 4 °C on plates, followed by 4-8 h white light induction. Subsequently, plates have been transferred to either complete darkness or exposed to various continuous FR (720 nm) fluencies: 5.3, 2.5, 0.5 µmol m^{-2} s^{-1} or 13 µmol m^{-2} s^{-1} when not indicated otherwise. For the analysis of hypocotyl length the 5 old-day seedlings have been laid to square plates on half-strength MS, 1% phytoagar for scanning. The hypocotyl length measurements have been performed analysing the scanned plated at resolution 300 dpi with Image J (http://rsb.info.nih.gov/ij/). All hypocotyl measurements were repeated at least three times; one representative experiment is shown.

Relative anthocyanin levels have been determined by collecting 60 seedlings from each of the light treatments/genotypes and incubating them overnight in 700 µl Extraction buffer prepared with a final concentration of 18% (v/v) 1-Propanol and 0.37% (v/v) HCl. In a further step the samples have been heated to 95 °C for 2 min and chilled on ice for 5 min. The tubes have been then incubated under continuous shaking overnight in the dark at 4 °C. On the next day a 10 minutes centrifugation step preceded the analysis of the supernatant. The total anthocyanin content has been determined by measuring the A_{535} and A_{650} using a spectrophotometer. The relative amount of anthocyanin per seedling has been calculated by subtracting the A_{650} from the A_{535} divided by the number of seedlings. The anthocyanin measurements show mean and standard deviation of three biological and technical replicates.

Microscopy

Image acquisition was performed with a Nikon ECLIPSE 90i microscope equipped with

YFP filters and a 64x water objective. The 4 day-old dark-grown seedlings (dark condition) were directly observed under the microscope using a safety green light. For the light conditions the 4 day-old dark-grown seedlings were pre-treated for 6 hours with FR light (720 nm, 13 µmol m^{-2} s^{-1}) either followed by a 5 min exposure to additional continuous R light (670 nm, 8 µmol m^{-2} s^{-1}) or not. All images were acquired using Metamorph (version 6.2r4). ImageJ (version 1.44k) and Photoshop (version 10.0.0.1) software was used for image processing.

Protein extraction and immunoblot analysis

Seedlings were grown in D on half-strength MS, 1% phytoagar. After 4 days, seedlings were harvested and used for extraction of total proteins as previously described (Kircher et al., 2002). Immunoblotting was done according to standard protocols. Commercially available GFP antibody (Covance) was used for detection of YFP-tagged proteins.

Figure Legends

Figure 1. An artificial FHY1 complements the *fhy1-3 fhl-1* mutant phenotype.

(A) FR-HIR for inhibition of hypocotyl elongation. Wild-type (Col-0), *phyA-211*, *fhy1-3 fhl-1, fhy1-3, fhl-1, fhy1-3* p35S:YFP-FHY1 and four different lines (#9526, #9537, #9742, #9717) of *fhy1-3 fhl-1* seedlings expressing p35S:NLS-YFP-FHY1 167-202 (artificial FHY1) were grown for 5 days in the dark or in different intensities of FR (0.5, 2.5, 5.3 µmol m^{-2} s^{-1}) before measurement of the hypocotyl length. Data are means relative to dark (D) controls (n>15); error bars indicate ±SEM. (B) FR-HIR for anthocyanin accumulation. The same transgenic lines as described in (A) were grown in the dark (D) or in continuous FR (FRc, 13 µmol m^{-2} s^{-1}) light. After 5 days the anthocyanin content was measured. The mean value (OD_{535}-OD_{650} / seedlings) of three technical replicates are shown; error bars indicate ±SD. (C) Subcellular localization of artificial FHY1. Four-day-old dark-grown *fhy1-3* p35S:YFP-FHY1 and *fhy1-3 fhl-1* seedlings expressing p35S:NLS-YFP-FHY1 167-202 (artificial FHY1) were analysed by fluorescence microscopy. The seedlings were analysed directly (dark), after 6 h irradiation with FR light (13 µmol m^{-2} s^{-1}) or after 6 h FR light exposure followed by 5 min of R light (8 µmol m^{-2} s^{-1}) (6 h FR + R). The scale bar represents 4 µm.

Figure 2. Constitutively nuclear localized phyA is functional *in vivo*.

(A) FR-HIR for inhibition of hypocotyl elongation. Col-0, *phyA-211*, *phyA-211 fhy1-3 fhl-1*, *fhy1-3 fhl-1*, *phyA-211* pPHYA:PHYA-NLS-YFP, *phyA-211 fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP (#10772, obtained by crossing *phyA-211* pPHYA:PHYA-NLS-YFP with *phyA-211 fhy1-3 fhl-1*; #9494, obtained by crossing *fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP with *phyA-211 fhy1-3 fhl-1*), *fhy1-3 fhl-1* pPHYA:PHYA Y242H-NLS-YFP and *phyA-211 fhy1-3 fhl-1* pPHYA:PHYA Y242H-NLS-YFP seedlings were grown in D or in continuous FR at 0.5, 2.5 or 5.3 µmol m^{-2} s^{-1}. After 5 days the hypocotyl length was measured. The mean hypocotyl length relative to dark controls are shown (n>15); error bars indicate ±SEM. (B) FR-HIR for anthocyanin accumulation. The anthocyanin content was measured for 5 days-old, dark- or FR- (FRc, 13 µmol m^{-2} s^{-1}) grown seedlings, as described in (A). The mean value (OD_{535}-OD_{650} / seedlings) of three biological and technical replicates are shown; error bars

indicate ±SD. (C) Light- and FHY1/FHL-independent nuclear localization of phyA-NLS. Seedlings of *phyA-211* pPHYA:PHYA-YFP*, fhy1-3 fhl-1* pPHYA:PHYA-YFP*, phyA-211* pPHYA:PHYA-NLS-YFP, *fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP, *phyA-211 fhy1-3 fhl-1* pPHYA:PHYA-YFP as well the two independent lines expressing pPHYA:PHYA-NLS-YFP in *phyA-211 fhy1-3 fhl-1* (#10772 and #9494) were gown for 4 days in darkness and analysed by fluorescence microscopy. The seedlings were analysed directly in Dark (D), after 6 h irradiation with FR light (13 $\mu molm^{-2}s^{-1}$) or after 6 h FR light followed by 5 min of R light (8 $\mu mol\ m^{-2}\ s^{-1}$) (6 h FR + R). The scale bar represents 4 µm. (D) In absence of FHY1/FHL, a constitutively nuclear localized phyA Y242H results in light-independent signalling. Col-0, *phyA-211 fhy1-3 fhl-1*, *fhy1-3 fhl-1* pPHYA:PHYA Y242H-NLS-YFP and *phyA-211 fhy1-3 fhl-1* pPHYA:PHYA Y242H-NLS-YFP seedlings were grown for 5 days in dark or in constant FR light (13 $\mu mol\ m^{-2}\ s^{-1}$).(E) FR-HIR for inhibition of hypocotyl elongation. Col-0, *phyA-211*, *fhy1-3 fhl-1* and *phyA-211 phyB*-9 *fhy1-3 fhl*-1 pPHYA:PHYA-NLS-YFP grown and analysed as in (A). Values of Col-0, *phyA-211*, *fhy1-3 fhl-1* as in Figure 1A. (F) FR-HIR for anthocyanin accumulation. Same Arabidopsis lines as described in (E).Anthocyanin content was measured for 5 days-old, dark- or FR- (FRc, 13 $\mu mol\ m^{-2}\ s^{-1}$) grown seedlings, as described in (B). Scale bar = 5 mm.

Figure 3. PhyA-NLS requires phyA downstream signalling components for normal FR responses

(A) Hypocotyl length measurements. 5 days-old seedlings grown as described in Figure 1 and 2 of the following Arabidopsis lines were used: Col-0*, phyA-211, phyA-211* pPHYA:PHYA-NLS-YFP, *hfr1-4*, *hfr1-4* p35S:HFR1*, phyA-211 hfr1-4* pPHYA:PHYA-NLS-YFP, *hy5-215*, *phyA-211 hy5-215* pPHYA:PHYA-NLS-YFP, *fhy1-3 fhl-1* and *fhy1-3 fhl-1* pPHYA:PHYA-NLS-YFP*.* Data show average hypocotyl length relative to dark controls (n>15); error bars indicate ±SEM. (B) Measurements of the anthocyanin content. Seedlings of the same lines as listed in (A) were grown for 5 days either in darkness or under continuous FR (FRc, 13 $\mu mol\ m^{-2}\ s^{-1}$). Data show mean values (OD_{535}-OD_{650} / seedlings) of three biological and technical replicates; error bars indicate ±SD. (C) Nuclear localization of pPHYA:PHYA-NLS-YFP in *phyA-211, phyA-211 hfr1-4* and *phyA-211 hy5-215* lines. Fluorescence microscopy images were taken as described in Figures 1C and 2C. Scale bar = 4µm.

Figure 4. PhyA-NLS expressing seedlings are strongly hypersensitive to R.

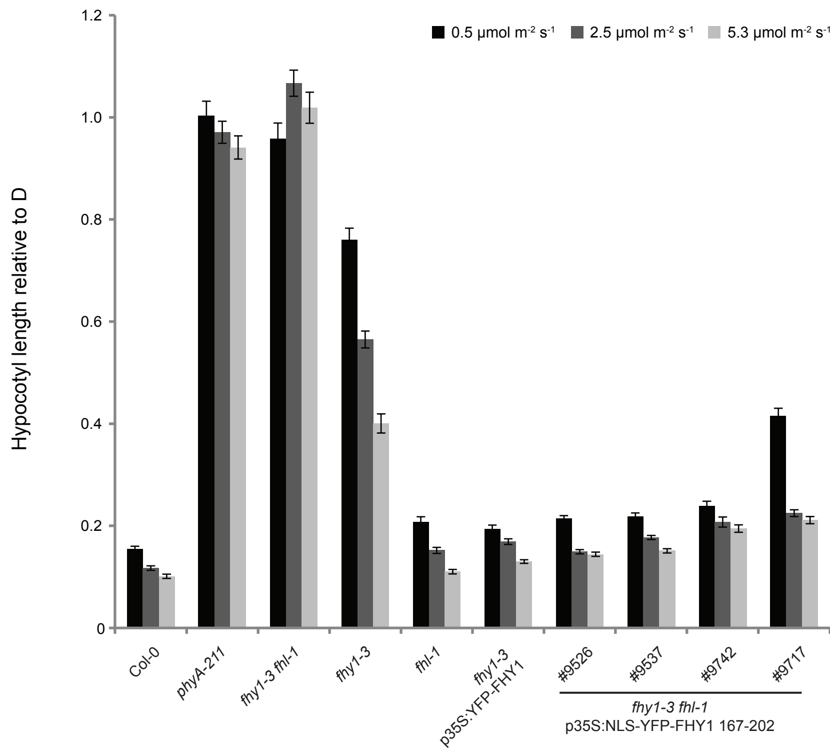
(A), (B) and (C). Fluence rate response curves for R and FR. Col-0, *phyA-211*, *phyB-9*, *phyA-211* pPHYA:PHYA-YFP, and *phyA-211* pPHYA:PHYA-NLS-YFP seedlings were grown for 4 day in R (A) or FR (B) of different fluence rates. Data show the mean hypocotyl length relative to D ($n \geq 20$); error bars indicate ±SEM. Part of the data shown in (A) and (B) are summarised in (C).

Figure 5. High expression levels of phyA-NLS-YFP result in constitutive photomorphogenesis.

Different light treatments (1, 2, and 3) were used to induce germination of Col-0 and *phyA-211* pPHYA:PHYA-NLS-YFP seedlings (three independent transgenic lines are shown). After induction of germination, seedlings were grown in D for 4 days and photographed. In addition, total proteins were extracted from dark-grown seedlings, separated on an SDS-PAGE and blotted onto nitrocellulose membrane. A GFP-specific antibody was used to detect YFP-tagged proteins.

Figure 1

A



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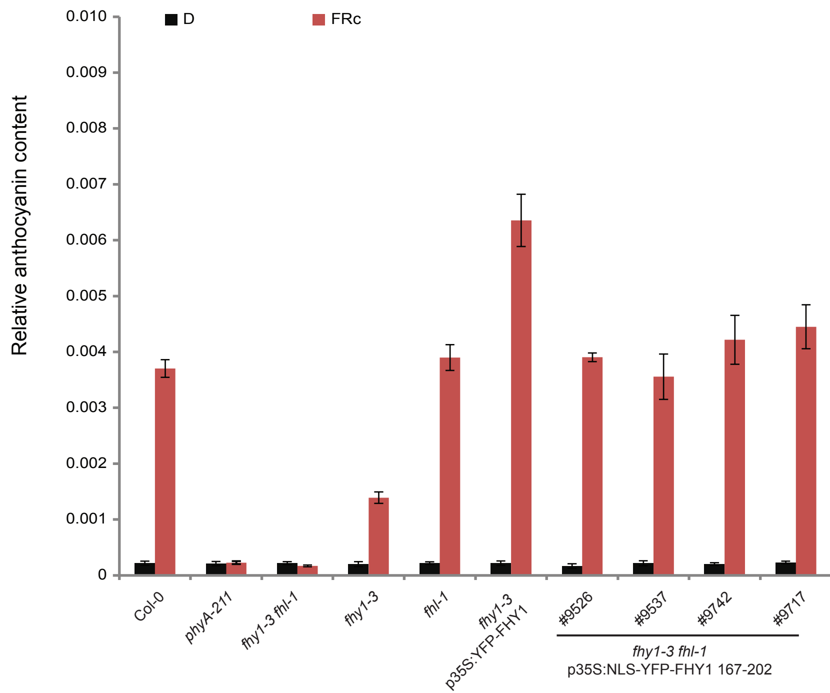
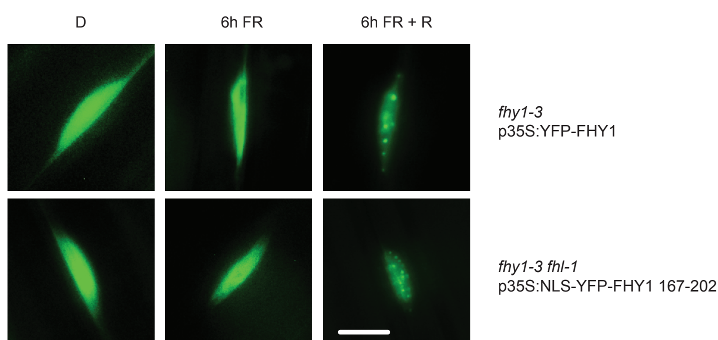


C



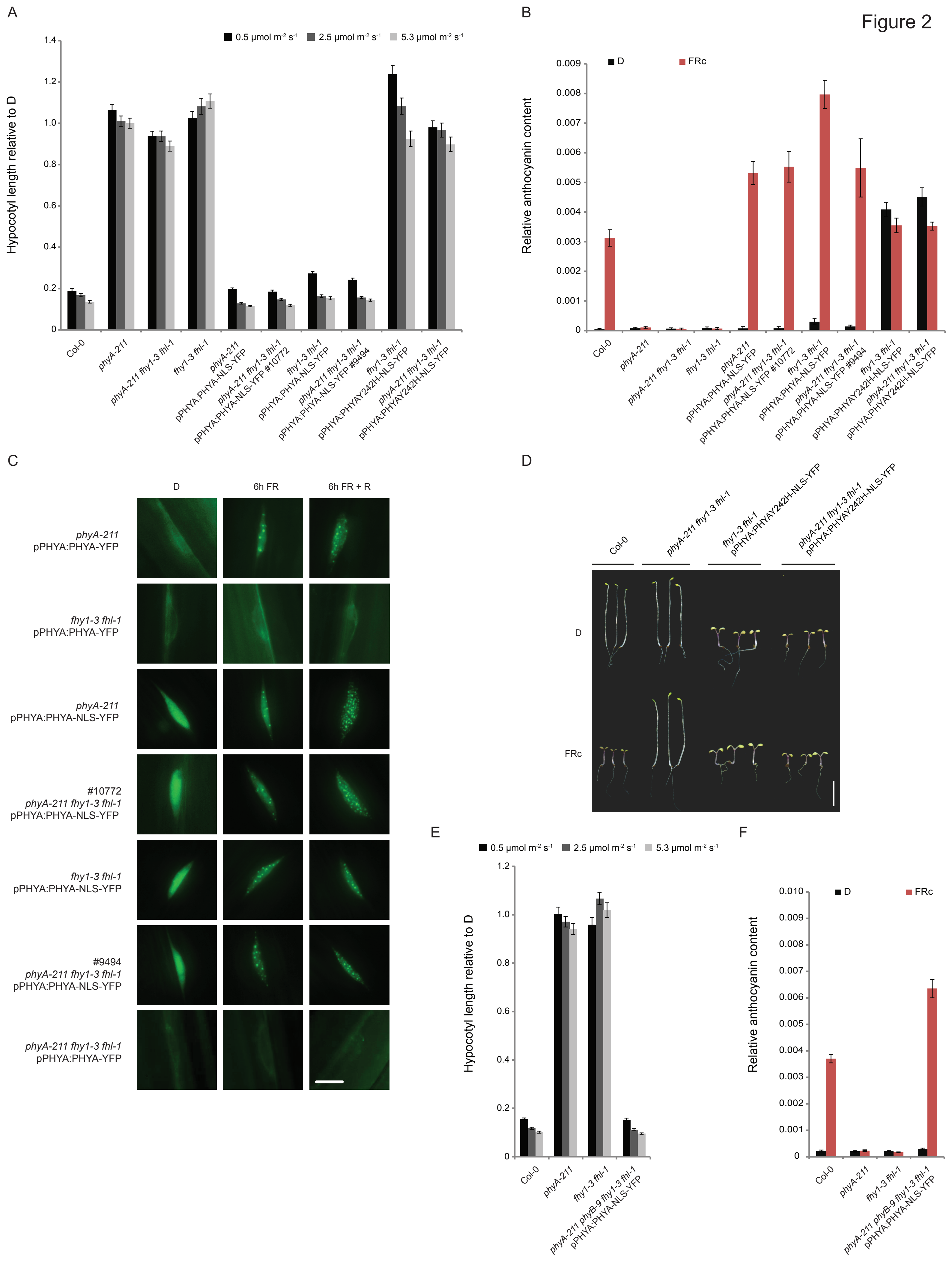
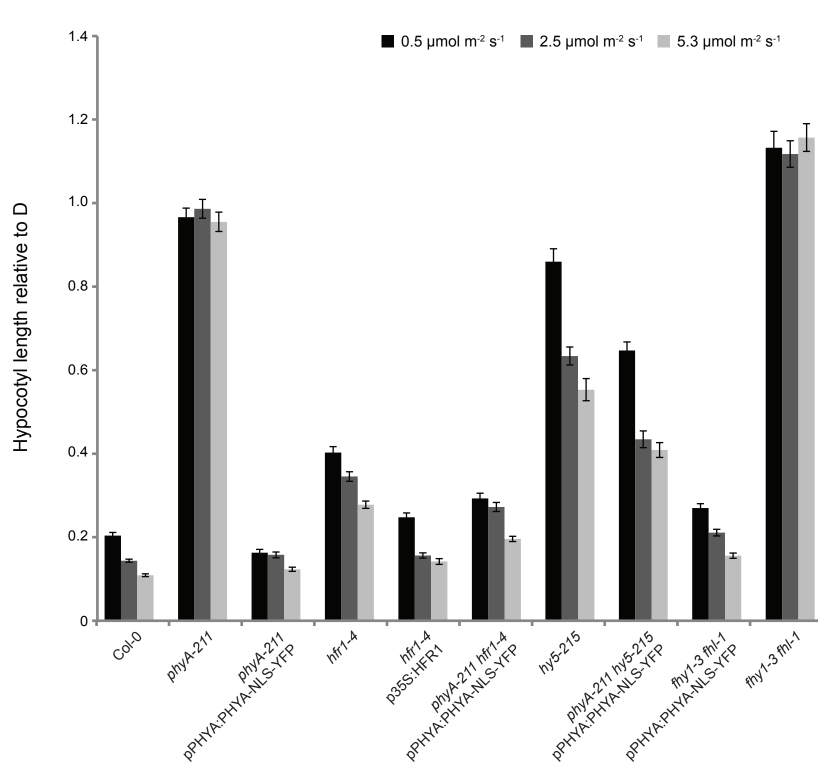
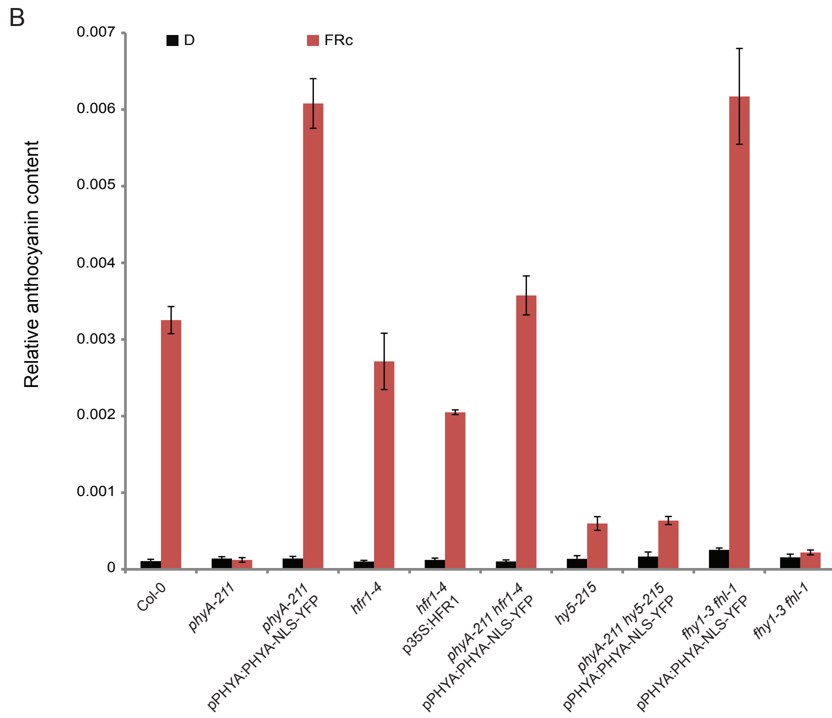


Figure 2
A
0.5 μmol m-2 s-1
2.5 μmol m-2 s-1
5.3 μmol m-2 s-1
Hypocotyl length relative to D
Col-0
phyA-211
phyA-211 fhy1-3 fhl-1
fhy1-3 fhl-1
phyA-211 pPHYA:PHYA-NLS-YFP
phyA-211 fhy1-3 fhl-1 pPHYA:PHYA-NLS-YFP #10772
fhy1-3 fhl-1 pPHYA:PHYA-NLS-YFP
phyA-211 fhy1-3 fhl-1 pPHYA:PHYA-NLS-YFP #9494
fhy1-3 fhl-1 pPHYA:PHYAY242H-NLS-YFP
phyA-211 fhy1-3 fhl-1 pPHYA:PHYAY242H-NLS-YFP
B
D
FRc
Relative anthocyanin content
C
D
6h FR
6h FR + R
phyA-211 pPHYA:PHYA-YFP
fhy1-3 fhl-1 pPHYA:PHYA-YFP
phyA-211 pPHYA:PHYA-NLS-YFP
#10772 phyA-211 fhy1-3 fhl-1 pPHYA:PHYA-NLS-YFP
fhy1-3 fhl-1 pPHYA:PHYA-NLS-YFP
#9494 phyA-211 fhy1-3 fhl-1 pPHYA:PHYA-NLS-YFP
phyA-211 fhy1-3 fhl-1 pPHYA:PHYA-YFP
D
Col-0
phyA-211 fhy1-3 fhl-1
fhy1-3 fhl-1 pPHYA:PHYAY242H-NLS-YFP
phyA-211 fhy1-3 fhl-1 pPHYA:PHYAY242H-NLS-YFP
D
FRc
E
0.5 μmol m-2 s-1
2.5 μmol m-2 s-1
5.3 μmol m-2 s-1
Hypocotyl length relative to D
Col-0
phyA-211
fhy1-3 fhl-1
phyA-211 phyB-9 fhy1-3 fhl-1 pPHYA:PHYA-NLS-YFP
F
D
FRc
Relative anthocyanin content
Col-0
phyA-211
fhy1-3 fhl-1
phyA-211 phyB-9 fhy1-3 fhl-1 pPHYA:PHYA-NLS-YFP

Figure 3





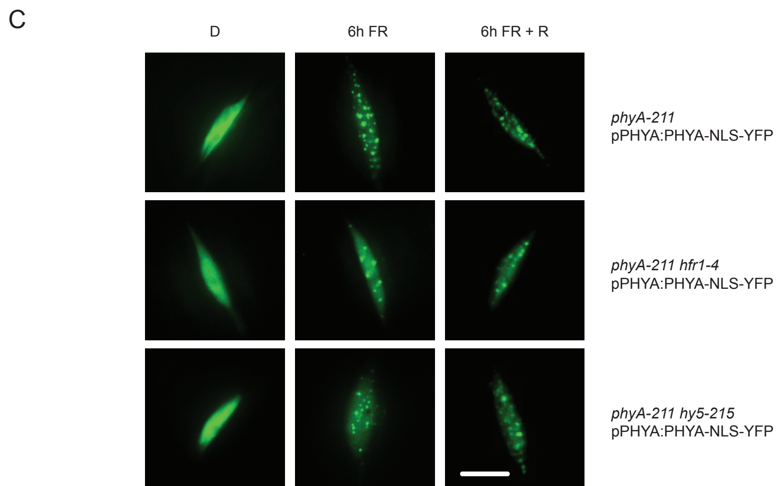


Figure 4

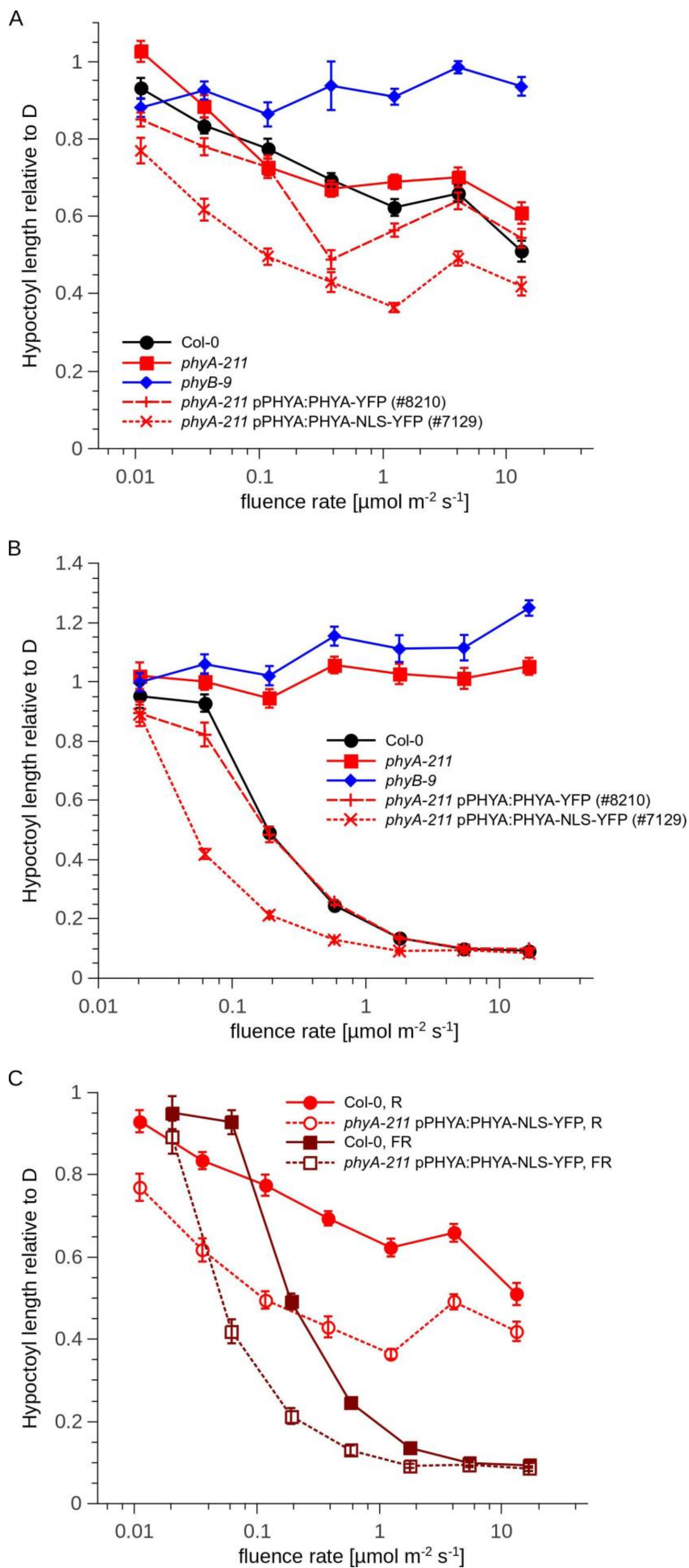
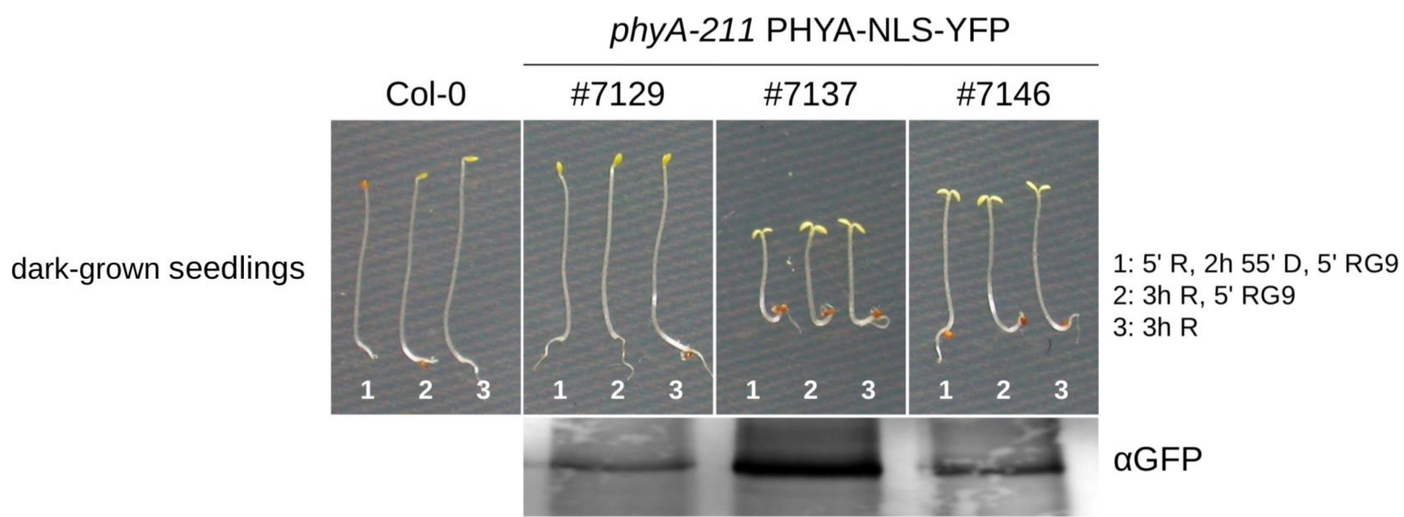


Figure 5



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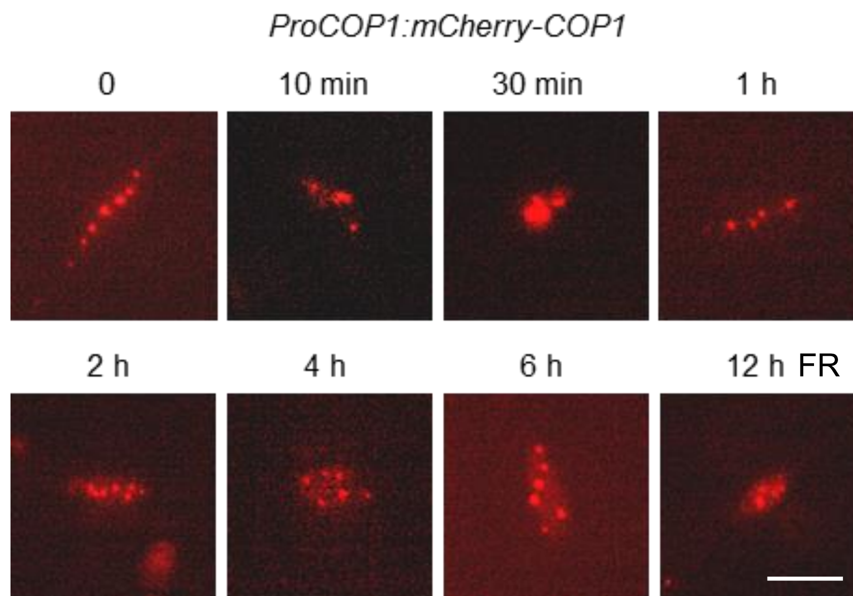
Author Contributions

AH, ES, CM, and CK designed research. CM, AH, and CK performed research and analyzed data. CM, AH, and CK prepared figures. AH and CM wrote the article.

Supplemental data



Supplemental Figure S1: FR do not affect COP1 nuclear-cytoplasmatic localization.

Time course and cellular localization of dark-grown Col-0 *ProCOP1:mCherry-COP1* Arabidopsis seedlings. The plants were exposed to increasing lengths of FR light (13 µmol $m^{-2}s^{-1}$) and analyzed by epifluorescence microscopy . Bar=5µm

CURRICULUM VITAE

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