Functional Analysis of the *SPA* Gene Family in *Arabidopsis thaliana*

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Kirsten Fittinghoff

aus Velbert

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Berichterstatter: Prof. Dr. Höcker

Prof. Dr. Hülskamp

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Contents:

Abbreviations

$\alpha\text{-}^{32}\text{P-dATP}$	2'-desoxyadenosin-5'-triphosphate, radioactive labelled at α-P-Atom
35S	35S promoter of Cauliflower Mosaic viruc
B	blue light
°C	degree Celsius
c	continous
µl	micro litre
µg	micro gram
bp	base pair
bHLH	basic helix-loop-helix
cDNA	complementary DNA
CC	coiled-coil structure
Col	Columbia; ecotype of *Arabidopsis thaliana*
cm	centimetre
cpm	counts per million
WD	aspartic acid
D	darkness
Da	Dalton
DNA	desoxyribonucleic acid
FR	far-red light
FRc	continuous FR
GUS	β-Glucuronidase
h	hour
HA	Influenza hemagglutinin
kb	kilo bp
kDA	kilo Da
l	litre
IR	infrared
LD	long day
Ler	Landsberg errecta; ecotype of *Arabidopsis thaliana*
M	molar; mol/l
mg	milligram
mM	millimolar
min	minute

mRNA	messenger-ribonucleic-acid
NLS	nuclear localization signal/sequence
nm	nanometre
ORF	open reading frame
PCR	polymerase chain reaction
Pfr	red light absorbing phytochrome conformation
Phy	phytochrome
Pr	red light absorbing phytochrome conformation
R	red light
RLD	ecotype of *Arabidopsis thaliana*
rRNA	ribosomal ribonucleic-acid
RNA	ribonucleic-acid
RT-PCR	reverse-transcription-PCR
s	second
SD	short day
UTR	untranslated region
U	unit
UV	ultraviolet
WD	aspartic acid; tryptophan
WS-0	Wassilewskaja; ecotype of *Arabidopsis thaliana*
WT	wild type
ZT	zeitgeber

Nomenclature:

SPA1	gene, locus, wild-type allele
spa1	mutant allele
SPA1	protein

Exception: photoreceptors

PHY	gene, locus, wild-type allele
phy	mutant allele
PHY	apoprotein (without chromophor)
phy	holoprotein (with chromophor)

Zusammenfassung

Viele Abschnitte im Lebenszyklus von Pflanzen, wie z.B. die Samenkeimung, die frühe Keimlingsentwicklung (De-etiolierung) oder die Induktion der Blütenbildung, werden maßgeblich durch das Sonnenlicht beeinflußt. Dabei spielt der Arabidopsis COP1-SPA Proteinkomplex eine zentrale Rolle, denn er verhindert eine Lichtantwort im Dunkeln. Der COP1-SPA-Komplex ist vor allem in Dunkelheit aktiv und verantwortlich für die Ubiquitin-vermittelte Degradation von positiv wirkenden Faktoren der Lichtsignaltransduktion, wie den Transkriptionsfaktoren HY5, der die Keimlingsentwicklung steuert, oder CONSTANS, der die Blütenbildung fördert. Im Licht wird die Funktion des COP1-SPA Komplexes gedrosselt, eine Aufgabe, die von mehreren Photorezeptoren bewerkstelligt wird. Das COP1 Protein wird in Arabidopsis von einem einzigen Locus kodiert, während die SPA Proteine von einer Genfamilie bestehend aus vier Mitgliedern kodiert werden (*SPA1-SPA4*). *SPA* Gene haben überlappende, jedoch auch distinkte Funktionen im Lebenszyklus von Arabidopsis. *SPA1* und *SPA2* sind hauptverantwortlich für die Unterdrückung der Photomorphogenese im Dunkeln. *SPA2* hat keine Funktion bei der lichtgesteuerten Keimlingsentwicklung, die hingegen hauptsächlich von *SPA1* und in geringerem Maße auch von *SPA3* und *SPA4* reguliert wird. *SPA1* ist zudem ausreichend, um eine verfrühte Blütenbildung im Kurztag zu verhindern.

Ziel dieser Arbeit war es, die molekularen Grundlagen der unterschiedlichen Funktionen der *SPA* Gene zu verstehen. Unterschiedliche *SPA* Gen-Funktionen lassen sich teilweise auf eine unterschiedliche *SPA* Genexpression zurückführen. RNA-Blot-Experimente zeigen, dass die mRNA-Mengen von *SPA1*, *SPA3* und *SPA4*, nicht aber die von *SPA2*, positiv durch Licht beeinflußt werden. Mehrere Photorezeptoren wirken dabei zusammen, um die Expression der *SPA* Gene im Licht unterschiedlicher Wellenlängen zu fördern. *SPA*-Promotor-Reportergen Analysen zeigen zudem eine zum Teil differentielle Expression der *SPA*-Gene während der Pflanzenentwicklung. Jedoch kann die unterschiedliche Expression der *SPA*-Gene nicht alle distinkten Funktionen der SPA Proteine erklären. Promoter-Austausch-Experimente mit den regulatorischen Elementen und cDNAs von *SPA1*, *SPA2* und *SPA4* zeigen, dass alle SPA Proteine im Dunkeln wirken können. SPA1 und SPA4 Proteine können außerdem im Licht als Repressoren fungieren, hingegen kann das SPA2-Protein nicht im Licht wirken, selbst wenn die *SPA2*-cDNA unter der Kontrolle

des licht-induzierten *SPA1*-Promotors steht. Diese Resultate zeigen, dass Unterschiede in der SPA Proteinsequenz ebenfalls einen Einfluß auf deren Funktionen haben.

Alle SPA Proteine zeigen eine ähnliche Domänen-Anordnung. Im stark konservierten carboxy-terminalen Bereich der SPA Proteine befindet sich eine WD-40 Repeat-Domäne, die ebenso wie die zentrale Coiled-Coil Domäne der SPA Proteine Interaktionen mit anderen Poteinen vermittelt. Die amino-terminale Region (N-Terminus) ist innerhalb der SPA Proteine weniger stark konserviert, zeigt aber in jedem der SPA Proteine eine schwache Ähnlichkeit mit einem Ser/Thr-Kinase-motiv. Um die Funktion dieser schwach konservierten Region näher zu untersuchen, wurde eine SPA1-Struktur-Funktionsanalyse durchgeführt. Interessanterweise ist ein SPA1 Protein ohne N-Terminus in der Lage, seine Rolle in der Keimlingsentwicklung vollständig auszufüllen. Hingegen ist es unfähig, die verfrühte Induktion der Blütenbildung im Kurztag zu hemmen. Daher zeigen diese Ergebnisse, dass der N-Terminus der SPA Proteine eine essentielle Rolle spielen kann.

Abstract

Ambient light conditions affect development throughout the plant life cycle, including seed germination, seedling development and the induction of flowering. In the model plant Arabidopsis, the COP1-SPA ubiquitin ligase complex plays a central role in suppressing light signaling in darkness. The COP1-SPA complex targets positively acting factors like HY5, a protein necessary for normal seedling development in the light, several photoreceptors and the flowering time regulator CONSTANS for degradation via the 26S proteasome. Therefore, one of the major functions of the light signal transduction pathways is the inactivation of the COP1-SPA complex. While *COP1* is a single copy gene, the SPA proteins are encoded by four different loci (*SPA1-SPA4*). All SPA proteins have redundant, but also distinct functions in regulating plant development. *SPA1* and *SPA2* are the key regulators that suppress photomorphogenesis in dark-grown seedlings. Over-stimulation in light-grown seedlings is primarily prevented by *SPA1*, and to a minor extent, also by *SPA3* and *SPA4*. SPA2, in contrast has only negligible function in the light. SPA1 is sufficient for repressing flowering under non-inductive short-day conditions.

Here, I show that distinct functions of the *SPA* genes partially correlate with their distinct gene expression patterns. RNA gel blot-analysis revealed that the expression of *SPA1*, *SPA3* and *SPA4*, but not that of *SPA2*, is positively influenced by light of different wavelengths. All main photoreceptors contribute to the up-regulation of these *SPA* transcripts, implying that photoreceptors initiate a negative feedback regulation, which might protect plants from over-stimulation by light. GUS reporter gene experiments show that *SPA* genes exhibit somewhat distinct tissue-specific expression patterns, which might be important for tissue specific regulation of COP1-SPA targets. However, differences in *SPA* gene expression cannot account for all distinct *SPA* gene functions. Promoter-swap experiments with *SPA1*, *SPA2* and *SPA4* show that all SPA proteins are potent repressors in dark-grown seedlings. SPA1 and SPA4 also act as repressor in the light. SPA2, however, can never act as a repressor in the light, not even when it is expressed from the strong light-induced *SPA1* promoter. These results show that SPA proteins themselves feature properties that determine characteristic SPA protein functions.

All SPA proteins feature a characteristic domain structure with a C-terminal WD-repeat, a central coiled-coil domain and a less well-conserved N-terminus that

includes a kinase-like motif. The WD-repeat domain and the coiled-coil domain are essential for formation of the COP1-SPA complex as well as interactions with various ubiquitination targets. In contrast, the function of the N-terminal domain is unknown. Aiming to determine the importance of the N-terminal domain of SPA1, I conducted a structure-function analysis. While the N-terminal domain of SPA1 is dispensable for SPA1 function in the seedling stage, this domain is required for SPA1-mediated repression of flowering in non-inductive short-day conditions. These results indicate, that the SPA1 N-terminal domain can full-fill an essential function.

I.   Introduction

I.1. Plant photoreceptors and light signal transduction

As sessile organisms, plants need to adopt their growth and development rapidly and optimally to ambient environmental changes. Light is not only the primary source of energy for plants, light is also an important environmental factor that influences many different developmental switches such as seed germination, seedling de-etiolation, shade avoidance, phototropism, stomata and chloroplast movement, circadian rhythm and induction of flowering. Seedling de-etiolation is one of the most drastic light responses. After germination, seedlings growing in the soil, i.e. in darkness, undergo a process called skotomorphogenesis in order to reach the soil surface and start photosynthesis. This is characterized by increased hypocotyl elongation, closed cotyledons and the formation of an apical hook, which protects the shoot apical meristem. On the soil surface seedlings are exposed to light and adopt their morphology for growing in the light. This developmental switch is called photomorphogenesis and is accompanied by inhibition of hypocotyl elongation, expansion of cotyledons and the induction of chlorophyll synthesis. This de-etiolation response in *Arabidopsis thaliana* seedlings has been used as a model system in forward genetic screens in order to identify photoreceptors and other regulatory factors important for light signaling.

In Arabidopsis, four main classes of photoreceptors are responsible for perceiving light of different intensities, qualities and directions (Briggs and Olney, 2001). Three different types of photoreceptors perceive blue light (B); three cryptochromes (cry1-cry3), two phototropins (phot1-phot2) and members of the zeitlupe gene family (ztl, lkp2, fkf1) (Ahmad and Cashmore, 1993; Lin et al., 1996; Huala et al., 1997; Christie et al., 1998; Mazzella et al., 2001; Kleine et al., 2003). phot1 and phot2 are involved in phototropic plant responses, chloroplast movement and stomatal opening (Briggs and Olney, 2001; Briggs and Christie, 2002; Sakamoto and Briggs, 2002; Ohgishi et al., 2004), whereas the ztl/lkp2/fkf1 photoreceptors regulate light input into the circadian clock and flowering time (Schultz et al., 2001; Imaizumi et al., 2003; Somers et al., 2004). cry1 is the primary photoreceptor that inhibits hypocotyl elongation in response to high fluence rates of B (Ahmad and Cashmore, 1993; Lin et al., 1996; Mazzella et al., 2001; Kleine et al., 2003). cry2 is important for seedling development under low fluence rates of B and plays an important role in the

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photoperiodic induction of flowering (Guo et al., 1998; Lin et al., 1998; Mockler et al., 2003).

The fourth class of photoreceptors, the phytochromes (PHYA-PHYE), monitor red light (R) and far-red light (FR) (Sharrock and Quail, 1989; Clack et al., 1994). phyA is the only photoreceptor that can sense FR, but in addition to that phyA can also mediate responses to low fluence rates of R and B (Nagatani et al., 1993; Whitelam et al., 1993). phyB and to a minor extent phyC, phyD and phyE play important roles in R response (Quail, 1997). Phys are known to regulate many different developmental steps such as seed germination, de-etiolation, shade avoidance and regulation of flowering time (Figure 1) (Schepens et al., 2004).
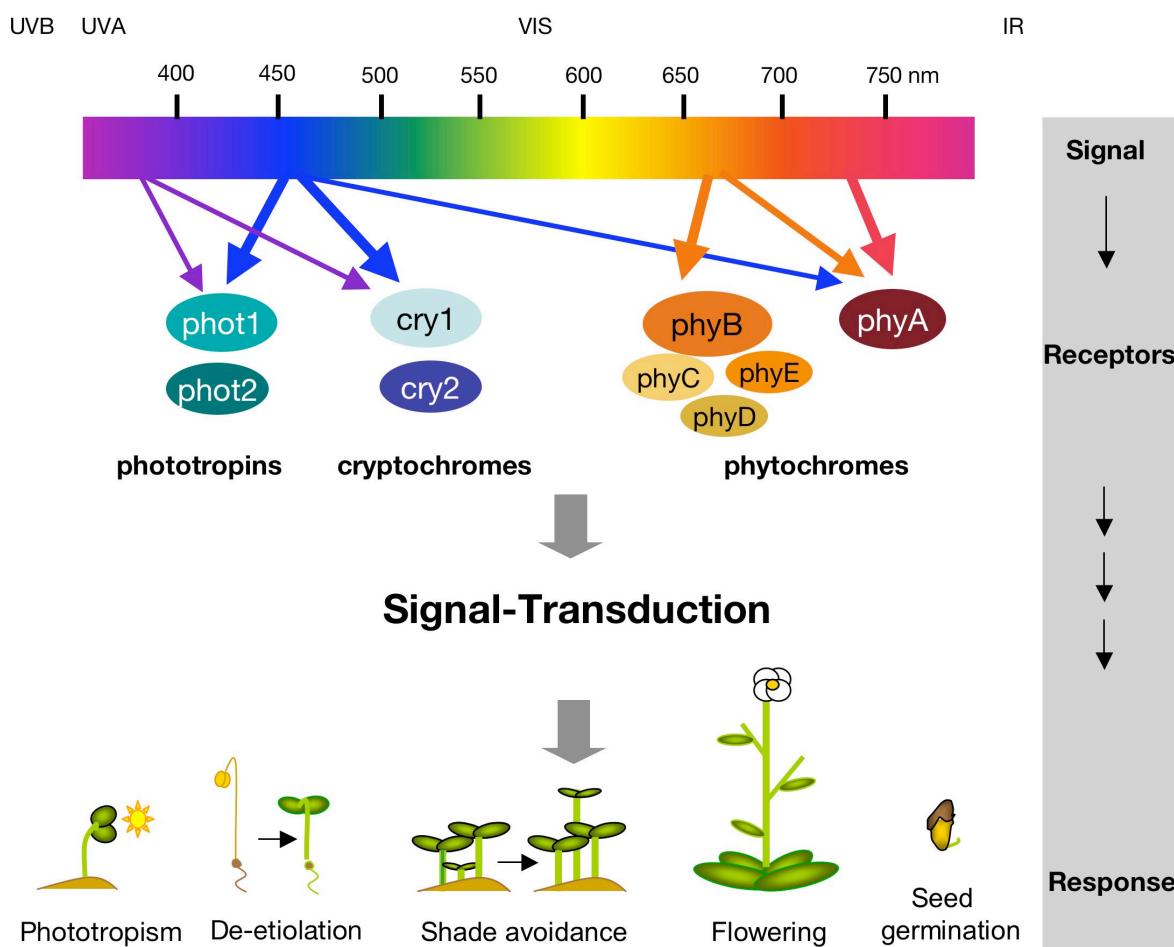
Figure 1: Role of photoreceptors during the plant life cycle.

Specialized classes of photoreceptors monitor light of different wavelengths. Cryptochromes and phototropins perceive B and UVA light. phyB mainly responds to R redundantly with phyA,C,D,E. phyA is the only FR-sensing photoreceptor but can also sense B and R. Photoreceptors modulate adaptive growth and development including seed germination, phototropism, de-etiolation, shade avoidance and induction of flowering.

Although Arabidopsis has evolved this sets of functionally distinct photoreceptors to monitor light, there is also vivid cross-talk between the different photoreceptors and their signaling pathways (Casal, 2000; Devlin and Kay, 2000; Mas et al., 2000; Mazzella et al., 2001; Yanovsky et al., 2001; Sullivan and Deng, 2003; Usami et al., 2004). In addition, the two major classes of photoreceptors, phys and crys, induce related signaling events. Once activated by light, phyA and cry2 become less stable and are degraded via the 26S proteasome pathway, while phyB and cry1 are stable also in the light (Guo et al., 1999; Hisada et al., 2000). Photo-activated receptors are mainly localized in the nucleus, the place where they initiate further downstream signaling events (Cashmore et al., 1999; Kircher et al., 1999; Kleiner et al., 1999; Yu et al., 2007). These signal cascades lead to a transcriptional reprogramming of the cells, which is coordinated by different classes of transcription factors. But how are these transcription factors regulated by the different photoreceptors? phys and crys follow two distinct strategies that directly and indirectly affect the activity of transcription factors involved in light signaling. First, photo-activated receptors can bind directly to some transcription factors. Phys physically interact with a class of bHLH transcription factors, so-called PHYTOCHROME-INTERACTING-FACTORS (PIFs) and PIF-LIKES (PILs). PIFs and PILs mainly act as repressors of light signaling and phytochromes can phosphorylate PIFs and PILs that are in turn degraded (Al-Sady et al., 2006; Castillon et al., 2007; Al-Sady et al., 2008; Leivar et al., 2008b; Leivar et al., 2008a; Shen et al., 2008). Similarly, photo-activated cry2 was recently shown to interact with the bHLH transcription factor CIB1 to regulate flowering time (Liu et al., 2008).

Several transcription factors with important roles in light signaling do not directly bind to photoreceptors. LONG HYPOCOTYL IN FAR-RED1 (HFR1), another bHLH transcription factor, is a component of phyA and cry1 signaling pathways and does not directly interact with phys (Fairchild et al., 2000; Duek and Fankhauser, 2003). Also LONG AFTER FAR-RED LIGHT1 (LAF1), a MYB transcription factor, does not directly bind phys but regulates gene expression in response to FR (Ballesteros et al., 2001). HYPOCOTYL 5 (HY5) and HY5 HOMOLOG (HYH), two bZIP-transcription factors, play a more widespread role in mediating light dependent transcriptional activation in seedling development under FR, R, B or UV-B light (Oyama et al., 1997; Chattopadhyay et al., 1998; Osterlund et al., 2000b; Osterlund et al., 2000a; Ulm et al., 2004). Common to HY5, LAF1 and HFR1 is that their regulation involves light-

dependent, post-translational control of protein stability: HY5, LAF1 and HFR1 proteins are low abundant in darkness and accumulate to high levels in the light (Osterlund et al., 2000b; Osterlund et al., 2000a; Seo et al., 2003; Duek et al., 2004; Jang et al., 2005; Yang et al., 2005b).
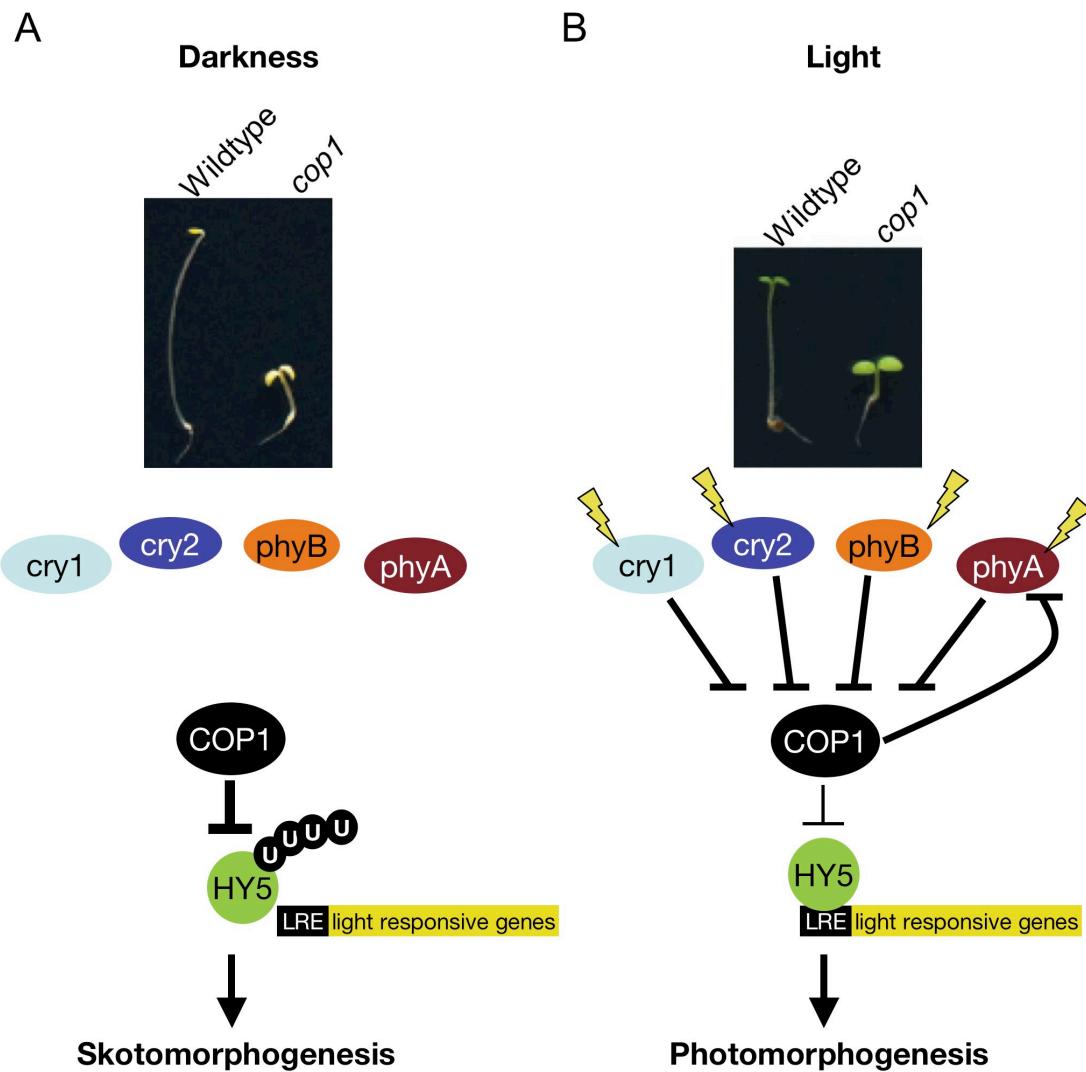
Figure 2: COP1 is a central regulator of light signal transduction.

A: Visual phenotypes of dark-grown wild-type and *cop1* mutant seedlings (top): In darkness wild-type seedling undergoes normal skotomorphogenesis showing long hypocotyl and closed cotyledons. *cop1* mutant seedling undergoes constitutive photomorphogenesis and exhibits the features of a light-grown seedling in darkness. Simplified illustration of molecular mechanism of skotomorphogenesis (bottom): In darkness, photoreceptors are inactive and cannot suppress negative regulators like COP1. In darkness, COP1 suppresses HY5 function, a transcriptional activator. Mutations in *COP1* lead to functional HY5 also in the darkness.
B: Visual phenotypes of light-grown wild-type and *cop1* mutant seedlings (top): In light wild-type seedlings exhibit reduced hypocotyls and de-etiolated (green) expanded cotyledons. *cop1* mutants show strong constitutive photomorphogenesis. Simplified illustration of molecular mechanism of photomorphogenesis (bottom): In light several photoreceptors suppress COP1 activity. In turn, HY5 protein becomes active and can activate transcription of light-responsive genes.

Photoreceptors promote the stability of these transcription factors indirectly by interfering with the factors that promote their degradation. These factors can be summarized in the group <u>CO</u>NSTITUTIVE <u>P</u>HOTOMORPHOGENESIS (COP), <u>DE</u>-<u>ET</u>IOLATED (DET) and <u>FUS</u>CA (FUS) proteins (Chory et al., 1989; Deng et al., 1991). Seedlings with mutations in any of the *COP/DET/FUS* genes exhibit short hypocotyls and open cotyledons in darkness (constitutive photomorphogenesis). The reason for this is that *cop/det/fus* mutants exhibit strongly elevated HY5 and HFR1 protein levels also in darkness (Osterlund et al., 2000b; Osterlund et al., 2000a; Seo et al., 2003; Duek et al., 2004; Jang et al., 2005; Yang et al., 2005b).

The most well characterized locus among the *COP/DET/FUS* genes is *COP1* (Deng et al., 1991). *COP1* encodes a protein with a carboxy-terminal WD-repeat domain, a coiled-coil domain and an amino-terminal RING motif, which is characteristic for one subclass of E3 ubiquitin ligases (Deng et al., 1992). In fact, COP1 has E3 ubiquitin ligase activity and targets the transcription factors HY5, HFR1 and LAF1 directly for degradation via the 26S proteasome (Osterlund et al., 2000a; Saijo et al., 2003; Seo et al., 2003). However, the molecular mechanism of photoreceptor-mediated inhibition of COP1 activity is not well understood.

The photoreceptors phyA, phyB, cry1 and cry2 can directly bind to the WD-repeat domain of COP1 and these interactions are thought to suppress COP1 activity towards other factors such as HY5 (Wang et al., 2001; Yang et al., 2001; Seo et al., 2004). Interestingly, COP1 seems in turn to be responsible for degradation of the light unstable phyA and probably also cry2 (Shalitin et al., 2002; Seo et al., 2004). COP1 becomes also inactivated by light-dependent exclusion from the nucleus, a process, which is also initiated by photoreceptor signaling (Von Arnim and Deng, 1994; Von Arnim et al., 1997; Subramanian et al., 2004). However, light does not completely suppress COP1 function, more likely trace amounts of biologically active COP1 remain in the nucleus to prevent over-stimulation by light.

COP1 genetically and physically interacts with other members of the COP/DET/FUS proteins (Schwechheimer and Deng, 2000). COP10, a ubiquitin activating E2 variant, interacts with both COP1 and also components of the COP9 signalosome, a multisubunit, nuclear protein complex involved in cullin-dependent ubiquitin/proteasome pathways (Wei et al., 1994; Yanagawa et al., 2004). COP10 itself forms a stable protein complex (the CDD complex) with DET1 and DDB1 that is thought to be important for COP1 activity (Yanagawa et al., 2004). Also, COP1 forms

high molecular weight complexes and interacts with several other proteins that are indispensable for COP1 function. One class of COP1-interacting proteins are the SUPPRESSOR OF PHYTHOCHROME A-105 (SPA) proteins (Hoecker and Quail, 2001; Laubinger and Hoecker, 2003; Saijo et al., 2003; Laubinger et al., 2004; Saijo et al., 2008; Zhu et al., 2008).
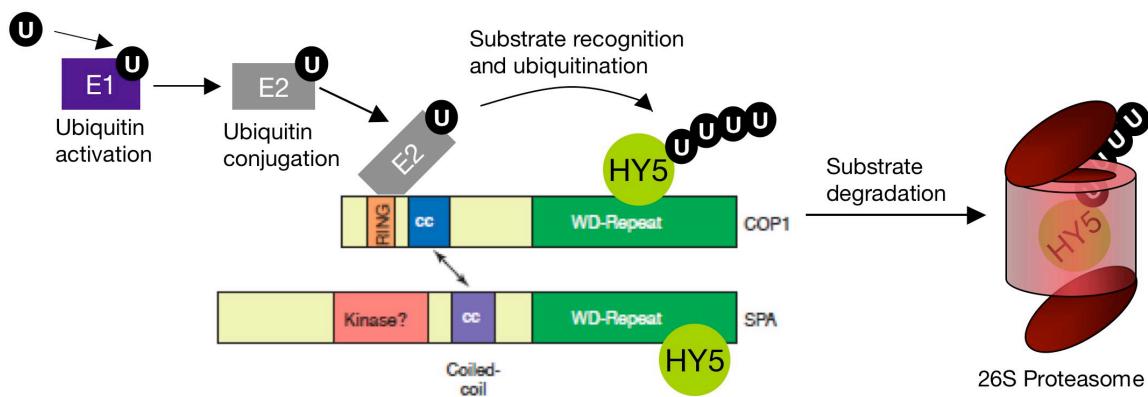
Figure 3: COP1 is an E3 ubiquitin ligase with structural similarities to SPA.

The ubiquitin-activating enzyme E1 binds and activates free ubiquitin (U) and transfers it to an ubiquitin-activating enzyme E2. After binding, the E2 ubiquitin-conjugating enzyme associates with COP1, an E3 ubiquitin ligase with a RING motif (typical for one class of E3 ubiquitin ligases). E3 ubiquitin ligases are responsible for substrate recognition. COP1 targets proteins by poly-ubiquitination for degradation via the 26S proteasom. COP1 shows structural similarity to the carboxy-terminal region of SPA including the WD-repeats. Transcription factors like HY5 can bind either the WD-repeat domain of COP1 or SPA1. Both proteins can physically interact through their respective coiled-coil domains (modified from Hoecker, 2005).

I.2. The SPA quartet: A family of COP1-interacting proteins with a central role in suppressing photomorphogenesis

The founding member of the *SPA* gene family, *SPA1*, was identified in a mutant screen for genes that suppress the phenotype of a weak *phyA* mutant allele (Hoecker et al., 1998). *spa1* mutants exhibit enhanced photomorphogenic responses in FR, R and B light, but are indistinguishable from wild-type seedlings in complete darkness (Hoecker et al., 1998; Baumgardt et al., 2002; Fittinghoff et al., 2006). The seedling phenotype of *spa1* mutants is only detectable in the presence of functional phyA, which led to the conclusion that SPA1 is a repressor of a phyA-specific signaling pathway (Hoecker et al., 1998). *SPA1* mRNA levels are strongly upregulated in response to R and FR, a process initiated not only by phyA, but also by phyB (Hoecker et al., 1999). *SPA1* encodes a constitutively nuclear-localized protein with three characteristic domains: a carboxy-terminal WD-repeat domain, a central coiled-coil domain and a N-terminal kinase-like domain (Hoecker et al., 1999). Within the

WD-repeat domain, SPA1 exhibit high sequence similarity to the WD-repeat domain of COP1 (Hoecker et al., 1999). The important relationship between COP1 and SPA1 function was corroborated by the observation that *spa1* and *cop1* mutations genetically interact, and that SPA1 is physically associated with COP1 in planta (Saijo et al., 2003). The interaction between SPA1 and COP1 is mediated by their respective coiled-coil domains and SPA1-binding influences the COP1 E3 ubiquitin ligase activity (Hoecker and Quail, 2001; Saijo et al., 2003; Seo et al., 2003; Saijo et al., 2008). The function of the N-terminal kinase-like domain of SPA1 is unknown and it remains to be elucidated whether the SPA1 protein exhibits kinase activity.

SPA1 is a part of a four-member gene family which includes three more members, *SPA1-related 2* (*SPA2*), *SPA1-related 3* (*SPA3*), and *SPA1-related 4* (*SPA4*; (Laubinger and Hoecker, 2003). All SPAs exhibit a similar domain architecture including a kinase-like motif, a coiled-coil domain and WD-repeats (Hoecker et al., 1999; Laubinger and Hoecker, 2003; Laubinger et al., 2004). Highest sequence similarity among all SPAs is found within their WD-repeat domains (Laubinger and Hoecker, 2003). SPA´s amino-termini including are less well conserved (22-27%). The *SPA* gene family can be divided into two subgroups. *SPA2* is most closely related to *SPA1* (Laubinger et al., 2004). *SPA1* and *SPA2* exhibit almost equal size and show conserved locations of all splice sites. The two members of the other SPA subgroup, SPA3 and SPA4, are highly conserved showing 74% identical amino acids (Laubinger and Hoecker, 2003).
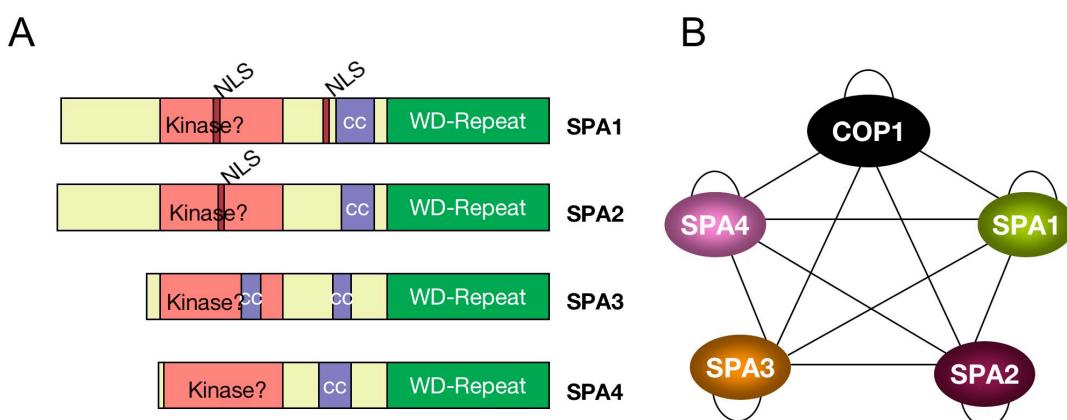
Figure 4: *SPAs* encode a small protein family that interacts with COP1.

A: All SPA proteins exhibit a carboxy-terminal WD-repeat domain and an amino-terminal kinase -like region. All SPAs feature at least one coiled-coil (CC) domain, which is known to mediate protein interaction or oligomerization. For SPA1 and SPA2 one or two nuclear localization sequences (NLS) are found.
B: All SPAs can form homo- and heterodimers with itself and other SPAs as well as COP1. COP1 can also form homodimers.

Reverse genetic approaches were conducted to uncover the role of *SPA2*, *SPA3* and *SPA4* in light-regulated plant development. *spa3* and *spa4* single mutants exhibit, like *spa1,* enhanced photomorphogenesis in FRc, Rc and Bc but are indistinguishable from wild type in the dark (Laubinger and Hoecker, 2003). Enhanced photomorphogenesis of *spa4* mutants, like that of *spa1*, mainly depends on functional phyA, whereas the *spa3* mutant phenotype might also depend on other phys (Laubinger and Hoecker, 2003). *spa2* single mutants do not show any obvious mutant phenotypes in the light when compared to the wild-type control (Laubinger et al., 2004). Because SPA proteins represent a protein family, it is possible that SPA protein have redundant functions that are partially masked when analyzing only *spa* single mutants. Indeed, *spa1 spa2 spa3 spa4* quadruple mutants undergo constitutive photomorphogenesis in darkness similar to a *cop1* mutant (Laubinger et al., 2004). This result indicates that all *SPAs* act redundantly in suppression of photomorphogenesis in the dark. These results are in agreement with the fact that all SPA proteins directly interact with COP1 and that the *spa2* mutant allele genetically interacts with the very weak *cop1^{eid6}* mutant allele (Laubinger et al., 2004). Recently, Zhu et al., 2008, showed that SPA proteins and COP1 form heterogeneous complexes in planta, possibly consisting of two COP1 and two SPA proteins. SPA proteins can form homo- as well as heterodimers depending on developmental stage and light regime (Zhu et al., 2008). Furthermore, COP1 complex formation is abolished in the absence of functional SPA proteins and vice versa, indicating that formation of COP1-SPA complexes is an essential step for COP1 and SPA protein function (Zhu et al., 2008).

I.3. Functional diversification among Arabidopsis *SPA* genes

Important results about the individual *SPA* gene functions were derived from a variety of *spa* double and triple mutants (Laubinger et al., 2004; Fittinghoff et al., 2006). *SPA1* and *SPA2* are both sufficient to prevent photomorphogenesis in darkness, while *SPA3* and *SPA4* play a rather minor role in regulating skotomorphogenesis (Laubinger et al., 2004; Fittinghoff et al., 2006). In light-grown seedlings, *SPA1* is the main player that suppresses photomorphogenesis (Laubinger et al., 2004; Fittinghoff et al., 2006). *SPA3* and *SPA4* also contribute to suppression of photomorphogenesis in the light, but the function of these two *SPA* genes is, when compared to *SPA1*,

rather dispensable and becomes only obvious when analyzing *spa3 spa4* double mutants (Laubinger et al., 2004; Fittinghoff et al., 2006). Interestingly, *spa3 spa4* double mutants show reduced adult plant size and the *spa* quadruple mutant shows dwarfism very similar to *cop1* mutants (Laubinger and Hoecker, 2003; Laubinger et al., 2004). A single *SPA3* or *SPA4* gene is almost sufficient for a normal adult growth, indicating that *SPA3* and *SPA4* play important roles in controlling adult plant size (Laubinger et al., 2004).
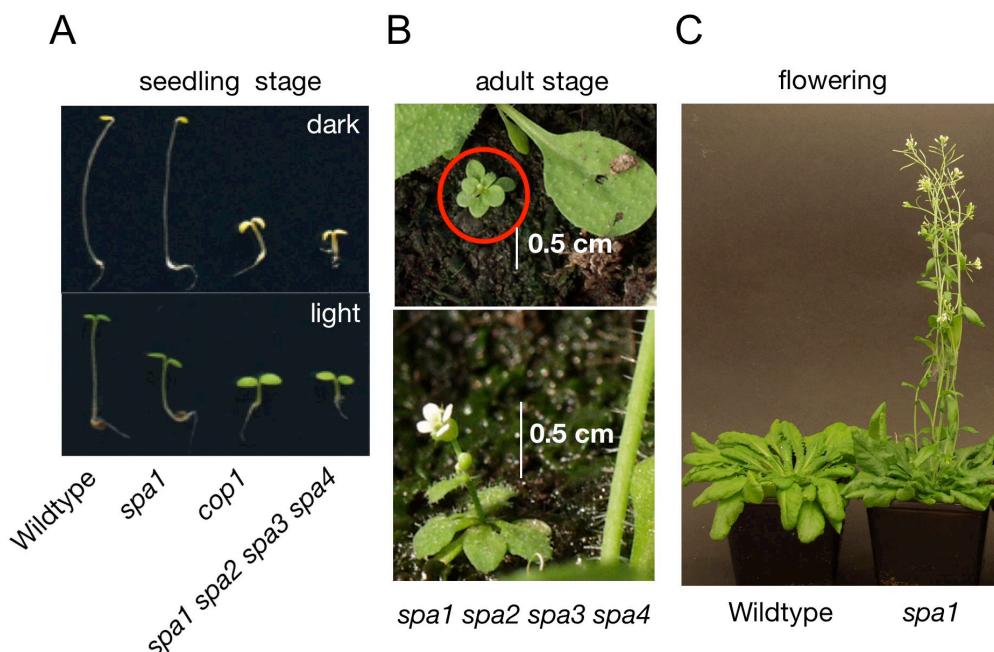
Figure 5: *SPAs* have redundant and also distinct functions in plant development.

A: Visual phenotypes of wild-type, *spa1*, *cop1* and *spa1 spa2 spa3 spa4* mutant seedlings grown in darkness (top) or under Rc (bottom). In darkness, wild-type plants exhibit long hypocotyls and closed cotyledons (skotomorphogenesis), whereas in the light hypocotyl length is reduced and cotyledons are open and green (photomorphogenesis). Mutations in *SPA1* result in enhanced photomorphogenesis in the light. *cop1* mutants exhibit constitutive photomorphogenesis in light and darkness. *spa1 spa2 spa3 spa4* mutant seedlings mimic the phenotype of the *cop1* mutant and show constitutive photomorphogenesis (pictures taken from Hoecker, 2005)
B: Visual phenotypes of a *spa1 spa2 spa3 spa4* adult plant which is strongly reduced in size (picture taken from Laubinger et al., 2004).
C: *spa1* mutants flower earlier in SD than wild-type plants (picture taken from Laubinger et al., 2006).

Another important, light-regulated step in the plant life cycle is the induction of flowering. *Arabidopsis thaliana* is a facultative long day (LD) plant that flowers early in long days and late in short days (SD) (Coupland et al., 1998). One major regulator of photoperiodic induction of flowering is *CONSTANS* (*CO*) (Putterill et al., 1995). *co* mutants flower late in LD whereas over-expression of CO leads to an early-flowering phenotype (Putterill et al., 1995; Onouchi et al., 2000). CO encodes a transcription

factor, which activates expression of <u>F</u>LOWERING LOCUS <u>T</u> (FT) and its homolog <u>TWIN-S</u>ISTER OF <u>F</u>T (TSF) (Koornneef et al., 1991; Yamaguchi et al., 2005). FT protein can move from the leaves to the shoot apical meristem where it interacts with the transcription factor FD to regulate expression of floral genes (Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al., 2007a; Mathieu et al., 2007).

Mutations in SPA1 cause an early-flowering phenotype in SD but not in long days (Ishikawa et al., 2006; Laubinger et al., 2006). Additional loss of SPA3 and SPA4 function further enhances the early-flowering phenotype of spa1 mutants (Laubinger et al., 2006). On the other hand, mutants that carry only a functional SPA1 gene flower like wild-type plants indicating that SPA1 alone is sufficient to repress flowering under SD conditions (Ishikawa et al., 2006; Laubinger et al., 2006). spa1 mutants accumulate high levels of FT mRNA while levels of CO are largely unaffected (Ishikawa et al., 2006; Laubinger et al., 2006). spa1 mutants flower only
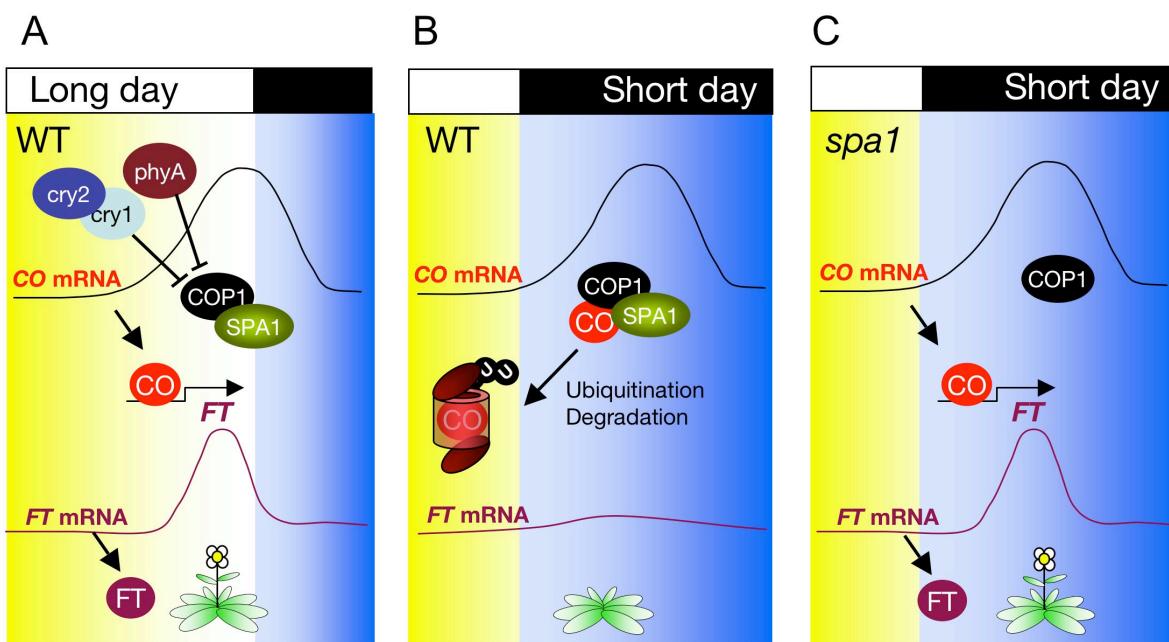
Figure 6: COP1/SPA complexes suppress flowering in SD by destabilization of CO.

A: CO mRNA levels in wild-type plants grown in long days (LD) accumulate in the end of late afternoon and coincide with light. Light stabilizes CO protein that activates FT mRNA transcription, an important inducer of flowering. Possible inactivation of COP1/SPA function could be due to physically interaction with photoreceptors like phys and crys.
B: In wild-type plants grown in short days (SD) the peak of CO mRNA levels occurs during night (darkness). COP1/SPA complex destabilize CO protein levels by targeting CO for degradation via the 26S proteasom. Without CO activation, FT mRNA levels are low abundant and flowering is not induced.
C: In spa1 mutant plants grown SD the peak of CO mRNA levels occurs during night (darkness). However CO is stable and activates FT transcription, which results in the earlier flowering phenotype of spa mutants in SDs.

early in SD in the presence of functional *CO* (Ishikawa et al., 2006; Laubinger et al., 2006). CO protein levels are strongly elevated in *spa1* and *spa1 spa2 spa3* triple mutants (Laubinger et al., 2006; Jang et al., 2008).

In addition, SPA1 interacts with CO in vitro and in planta, raising the possibility that CO is an ubiquitination target of the COP1-SPA complex (Ishikawa et al., 2006; Laubinger et al., 2006). This is in agreement with the observation that also *cop1* mutants flower early in SD, that CO protein accumulates in a *cop1* mutant, that COP1 interacts with CO and that COP1 ubiquitinates CO in vitro (Laubinger et al., 2006; Jang et al., 2008).

Taken together, the COP1-SPA complexes play important roles in many different developmental stages. It seems that the contribution of the individual *SPA* genes differs in each developmental stage. *SPA1* can suppress photomorphogenesis in the dark and the light and also regulates photoperiodic induction of flowering. *SPA2* function is limited to dark-grown seedling and it has only very minor functions in later developmental stages that are influenced by light. *SPA3* and *SPA4* only have minor functions in dark- and light-grown seedlings, but they play important roles in regulating adult plant size.
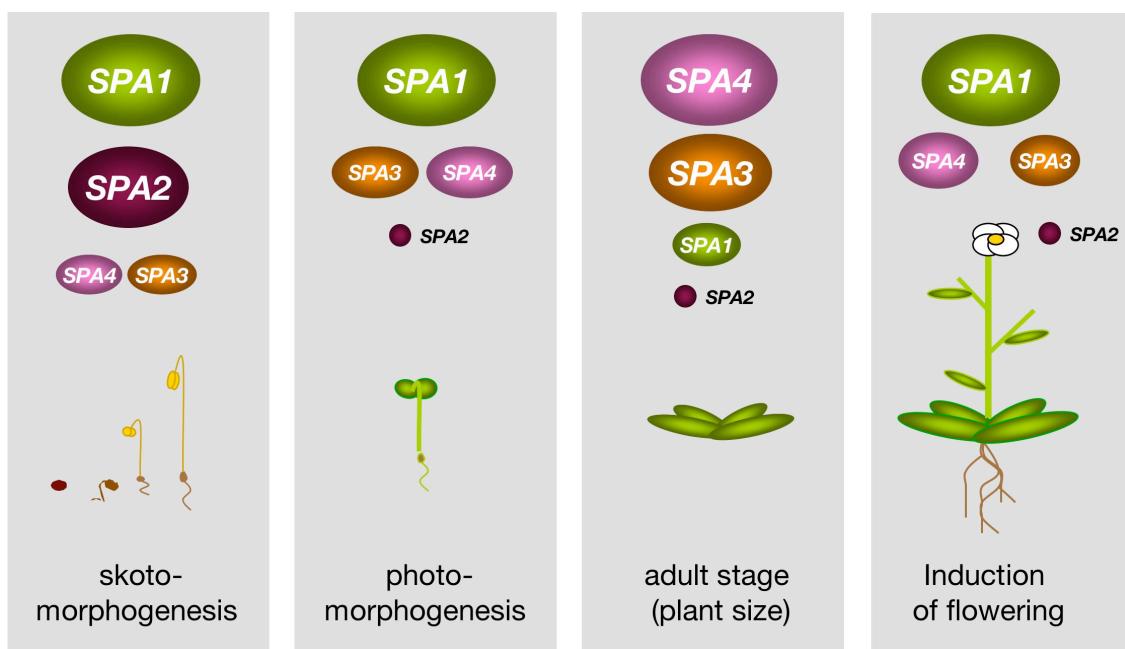
Figure 7: SPA proteins have redundant but also distinct functions in regulating plant development.

SPA1 and *SPA2* predominate in suppressing photomorphogenesis in darkness, whereas *SPA3* and *SPA4* play only minor roles in this developmental stage. *SPA1*, and to minor extend *SPA3* and *SPA4*, repress photomorphogenesis in the light. *SPA3* and *SPA4* are the most important *SPA* genes regulating adult plant size. *SPA1* is sufficient for preventing early flowering in non-inductive short days (SD).

I.4. Aims of this PhD thesis

Previous genetic analyses have shown that the four *SPA* genes have partially distinct functions in the control of seedling development in light/darkness, plant size and photoperiodic flowering. This thesis, therefore, aims to identify underlying molecular mechanisms for the functional diversification among *SPA* genes. Conceptually, differential *SPA* gene function might be caused by differences in *SPA* expression levels during development and/or differences among SPA protein sequences. These hypotheses are tested by:

(i) determining *SPA* transcript levels during development and in light vs. darkness

(ii) analyzing tissue-specificity of *SPA* expression by examining *SPA-promoter::GUS* transgenic plants

(ii) conducting promoter/cDNA swaps among *SPA* genes

The second aim of this thesis addresses a structure-function analysis of SPA1. While *spa1* mutant alleles have indicated a functional requirement for the C-terminal WD-repeat domain, little was known about the N-terminal domains of SPA1. Therefore, N-terminal deletion-derivatives of SPA1 are generated and tested for their ability to complement the *spa1* mutant phenotype.

II. Results

II.1. SPA1 structure-function analysis

Genetic analysis of diverse multiple *spa*-mutants showed that *SPAs* have overlapping but also distinct functions in regulating plant development. Based on their function and sequence similarity, SPA proteins can be divided into two classes. SPA1 and SPA2 proteins are closely related and both important to inhibit photomorphogenesis in dark-grown seedlings (Laubinger et al., 2004). SPA3 and SPA4 proteins share up to 85% identical amino acids and both are mainly involved in regulating growth of adult plants (Laubinger and Hoecker, 2003). All SPA proteins feature a similar protein domain arrangement: High similarity among all SPA proteins is found in their C-terminal regions that include WD-repeats, an important protein domain that is also characteristic for central repressor of light signaling, COP1. For SPA1 and COP1 it was shown that WD-repeats are essential for binding transcription factors like HY5 or HFR1 (Hoecker and Quail, 2001; Saijo et al., 2003; Yang et al., 2005a). All SPAs carry at least one or two predicted coiled-coil domains, which are known to mediate homo- or heterodimerization. Indeed, the predicted coiled-coil regions of SPA proteins are essential for binding COP1 as well as other SPAs (Hoecker and Quail, 2001; Laubinger and Hoecker, 2003; Saijo et al., 2003; Zhu et al., 2008).

While the role of the WD-repeat domain and the central coiled-coil domain of the SPA proteins is well established, the function of the N-terminus is completely unknown. Although all SPA proteins exhibit similarity with serin-/ threonin- kinases in their N-terminus, it is the most unconserved region within the different SPA proteins. In addition, the N-terminus of *SPA1* and *SPA2* is much longer than that of *SPA3* or *SPA4* and carries two putative nuclear localization sequences (NLSs).

II.1.1. SPA1 N-terminus is not required for SPA1 function in dark- and light-grown seedlings, whereas the coiled-coil domain is essential

To examine if redundant and non-redundant *SPA* functions are based on differences in SPA protein structure, especially in the unconserved N-terminal region, it is important to know which structure is relevant for SPA1 function. To test whether the coiled-coil, the kinase-like domain or the whole N-terminus is important for SPA1 function in vivo, *SPA1* deletion constructs were generated (Figure 8A): *ΔN* lacks most

of the *SPA1* N-terminus, *ΔKIN* lacks only a smaller part of the N-terminus, which contains highest sequence homology among the four SPA proteins (Laubinger and Hoecker, 2003). Another deletion-construct produces a SPA1 protein without the predicted coiled-coil domain (Δcc; Hoecker et al., 1999). As a positive control, the *SPA1* cDNA coding for the full-length SPA1 protein was used (FL SPA1). The *SPA1* deletion-derivates and the full-length cDNA were fused to a sequence encoding a triple influenza hemagglutinin (*3xHA*). All described constructs (IV.3.3 and Figure 8 A) were placed under the control of *SPA1* endogenous 5′ (-2241 base pairs upstream of the SPA1 start codon) and 3′(1026 base pairs downstream of the stop codon) regulatory sequences.

To test which domain is necessary for *SPA1* function all deletion-derivates and the full length *SPA1* cDNA were transformed into *spa1-3* mutant plants. Mutant *spa1-3* seedlings show an enhanced de-etiolation in response to FRc with characteristic short hypocotyls and fully opened cotyledons (Hoecker et al., 1998; Figure 8 A). Therefore, transgenic *spa1-3* seedlings were analyzed under fluence rate of FRc.

The vast majority of all investigated transgenic T2 lines carrying FL SPA1, ΔN or ΔKIN deletion-derivates of SPA1 showed segregated seedlings with long hypocotyls and partially closed cotyledons in low FRc, like WT seedlings (Figure 8 B). Thus, expression of FL SPA1 or either its deletion-derivates ΔKIN or ΔN in *spa1-3* mutants fully restored the WT phenotype (Figure 8 B). Hence, deletion of SPA1 N-terminus did not affect SPA1 protein function. Because *SPA1* is also important for suppression of photomorphogenesis in darkness, *SPA1* N-terminal deletion constructs were also transformed into *spa1 spa2 spa3* triple mutants that show photomorphogenesis also in complete darkness. SPA1 proteins that lack either the kinase domain or the whole N-terminus fully rescued the phenotype of *spa1 spa2 spa3* triple mutants indicating that the N-terminus of *SPA1* is dispensable for *SPA1* function also in darkness.

To be able to statistically quantify photomorphogenesis in the transgenic lines, complementing lines carrying single insertions were propagated to non-segregating T3 plants that are homozygous for the transgene. Hypocotyl lengths of around 30 seedlings of each T3 line were measured to determine complementation efficiency. Measurements of two independent transgenic T3 lines for each construct showed that hypocotyls of FR-grown *spa1* mutants carrying *FL-SPA1*, *ΔKIN* or *ΔN* deletion-derivates were as tall as those of WT. This results show that the SPA1 C-terminus including the predicted coiled-coil domain and the WD-repeats is sufficient to fully

rescue the *spa1-3* mutant phenotype (Figure 8 D). Moreover, these results indicate that the putative NLS located in the SPA1 N-terminus, which is also deleted in ΔN transgenic lines, is not necessary for SPA1 function. If nuclear localization is necessary for SPA1 function these results suggest that the second NLS motif (KKKKASK) is sufficient for SPA1 function.
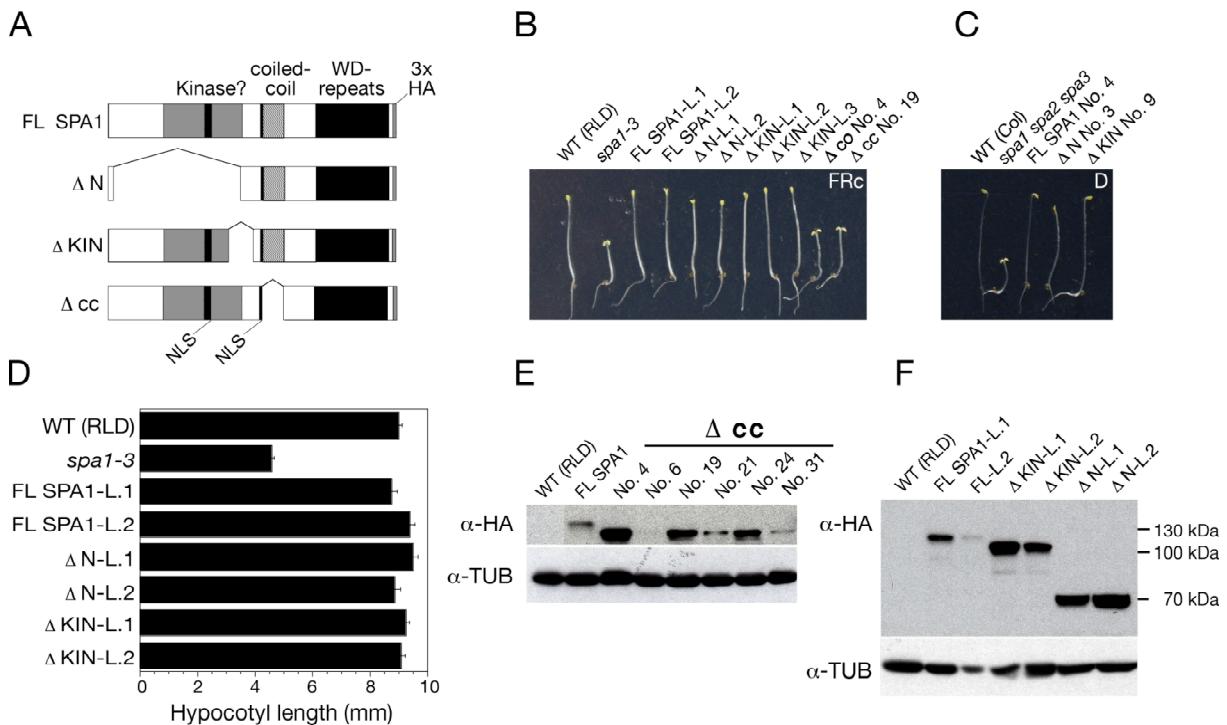
Figure 8: The N-terminal domain of SPA1 is not required for SPA1 function, whereas the coiled–coil domain is essential.

A: Schematic representation of full-length SPA1 (FL SPA1) and three SPA1 deletion mutants tagged with 3xHA. All constructs are under the control of endogenous *SPA1* 3´and 5´ regulatory elements.
B, **D**: Visual phenotypes (B) and hypocotyl lengths (D) of wild-type (WT), *spa1-3* and transgenic *spa1-3* seedlings that were transformed with *FL SPA1* or *SPA1* deletion constructs shown in A. For each construct two independent transgenic lines are shown. For complementing lines in *spa1-3* mutant background established non-segregating T3 generation are shown (L1 and L2). Non-complementing lines are shown in segregating T2 generation and presented with numbers (Δcc No.4). Seedlings were grown in 0.3 µmol m^{-2} s^{-1} FRc for 3 days. Error bars in D denote one standard error of the mean.
C: Visual phenotypes of dark-grown wild-type (WT), *spa1 spa2 spa3* and transgenic *spa1 spa2 spa3* seedlings containing FL SPA1, Δ N or Δ KIN deletion derivates, respectively.
E, F: Immunoblot analysis of transgenic *spa1-3* seedlings transformed with Δ cc in T2 generation (E), FL SPA1, Δ N or Δ KIN constructs in T3 generation (F). Seedlings were grown for 3 days in 0.3 µmol m^{-2} s^{-1} FRc. For immunodetection the membranes were incubated with an α -HA antibody and subsequently rehybridized with an α -tubulin antibody.

On the contrary, *spa1-3* seedlings expressing a SPA1 protein lacking the coiled-coil domain (Δcc) showed short hypocotyls and fully expanded cotyledons in FRc, like the *spa1* mutant progenitor (Figure 8 B). Out of 39 analyzed transgenic lines none

showed any rescue of the *spa1* mutant phenotype. To verify that those non-complementing transgenic lines indeed expressed the Δcc SPA1 deletion-protein, six randomly selected T2 lines were chosen for immunoblot analysis. Five lines showed detectable amounts of expressed Δcc SPA1 protein. Therefore I conclude that the central coiled-coil domain is essential for *SPA1* function. Western-blot analysis of complementing T3 lines expressing FL SPA1 showed that the amount of expressed SPA1 differs between the two lines tested. Even weaker expression of FL SPA1 protein was sufficient to rescue the *spa1-3* mutant phenotype. The SPA1 deletion-proteins ΔKIN and ΔN were also detectable and in both analyzed T3 lines more abundant than FL SPA1 (Figure 8 F).

These results show that the SPA1 N-terminus including the potential kinase-like domain is not necessary for SPA1-dependent inhibition of photomorphogenesis in dark- or light-grown seedlings. In contrast, the coiled-coil domain is essential for *SPA1* function.

II.1.2. SPA1 N-terminus is required to suppress flowering in short-days

Apart from suppression of photomorphogenesis in seedlings, *SPA1* also plays an important role in the regulation of flowering time. *spa1* mutants flower earlier than WT under SD conditions, but not under LD conditions (Laubinger et al. 2006; Ishikawa et al., 2006). Recent studies show that SPA1 and COP1 suppress flowering in SD by destabilizing CO, an important regulator of photoperiodic induction of flowering time (Laubinger et al. 2006; Jang et al.2008).

To investigate whether the SPA1 N-terminal region is important to suppress flowering in short days, 10 to 15 plants of two independent *spa1-3* T3 lines each carrying of FL SPA1, ΔN or ΔKIN were grown under SD conditions (eight hours light and 16 hours darkness). To determine flowering time the rosette leaves were counted at the time plants started bolting (Figure 9 A). Transgenic *spa1-3* plants expressing FL SPA1 started to flower almost as late as the WT, indicating that FL SPA1 complemented the *spa1* phenotype. In contrast, expression of ΔN in *spa1-3* mutants was not able to rescue the *spa1* mutant flowering time phenotype. These plants flowered as early as *spa1-3* mutants in short days. Plants carrying *Δ KIN* deletion-derivate flowered slightly later than *spa1-3* mutants, indicating that the ΔKIN deletion-protein has some residual function.
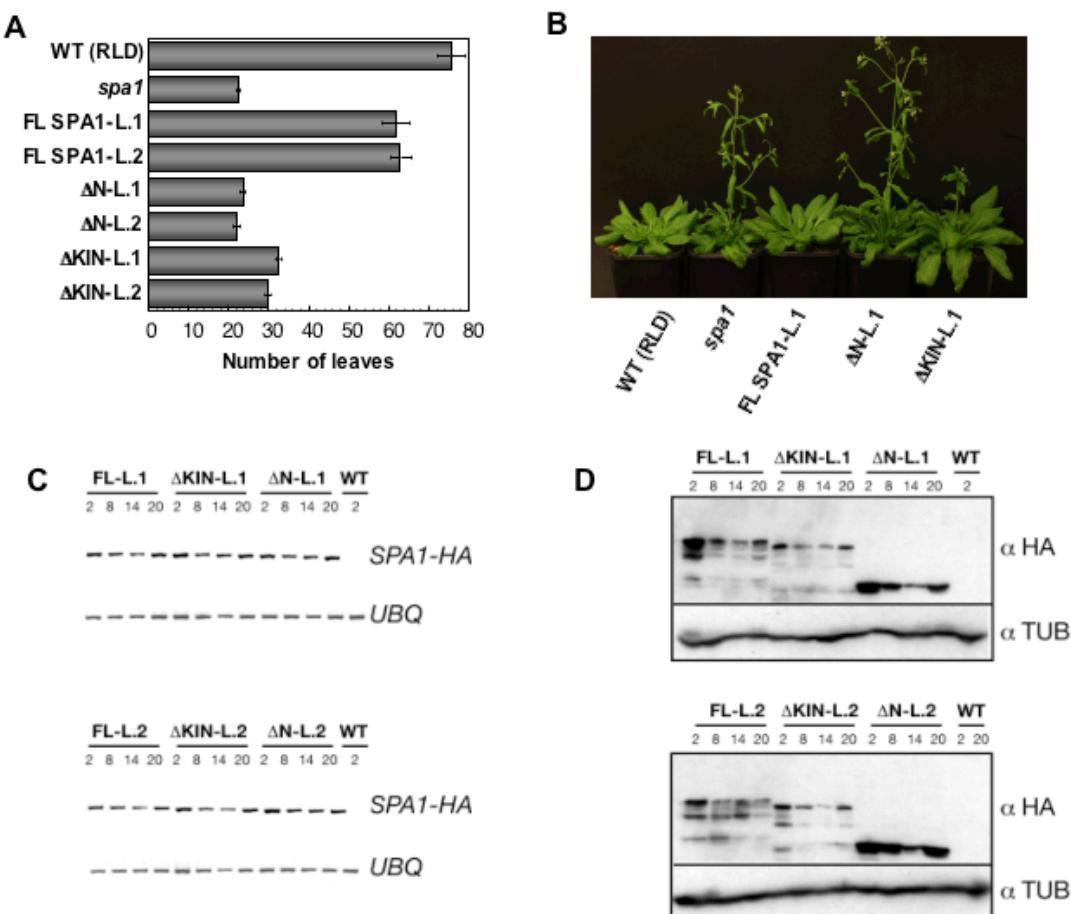
Figure 9: SPA1 N-terminus is necessary to inhibit photoperiodic induction of flowering in SD.

A: Flowering time in SD of two independent lines of genotypes shown in Figure 8 A.
B: Visual phenotypes of 78 days-old wild type (WT), *spa1-3* mutants and *spa1-3* mutants transformed with *FL SPA1*, *Δ N* or *Δ KIN* grown in SD.
C: Semi-quantitative RT-PCR of *SPA1-HA* and *UBQ10* transcript in 21 days-old plants grown in SD (8 hours light followed by 16 hours darkness) and harvested at Zeitgeber 2, 8, 14, 20.
D: Immunoblot analysis of 21 days-old wild-type (WT), *spa1-3* plants transformed with *FL SPA1 (FL)*, *Δ KIN* or *Δ N*. For each construct two independent non-segregating T3 lines were analyzed. All plants were grown in SD and harvested at same Zeitgeber described in C. For immunodetection the membranes were incubated with an α -HA antibody and subsequently re-hybridized with an α -tubulin antibody.

The circadian clock influences flowering time and various genes involved in photoperiodic flowering are regulated in a diurnal or circadian fashion. Also for *SPA1* a diurnal and circadian regulation was reported (Harmer et al., 2000; Ishikawa et al., 2006; Laubinger et al., 2006). To investigate whether deletion-derivates show proper diurnal regulation, transcript levels of *FL SPA1* and its deletion-derivates *ΔN* and *ΔKIN* at were analyzed different time points of the day (Zeitgeber, ZT). On transcriptional levels, no differences in diurnal regulation were observed between the mRNA of *FL SPA1* and the mRNA of the deletion-derivates *ΔN* and *ΔKIN* (Figure 9).

All lines showed a slight increase of transcript abundance at ZT 2 and ZT 20 (Figure 9 C). On the protein level, FL SPA1 and the truncated SPA1 proteins showed highest protein abundance at ZT 2 and ZT 20. ΔN SPA1 deletion-proteins accumulated to higher levels than FL SPA1 but showed similar diurnal pattern (Figure 9 D).

Taken together, the N-terminus of SPA1 and the sequence including the kinase-like motif are important for SPA1 function in the control of flowering time. RT-PCR and immunoblot-analyses showed that *SPA1* deletion-derivates do not exhibit an altered diurnal expression pattern on either transcriptional or protein levels.

II.2. *SPA* transcript analyses

Results of *SPA1* structure-function analysis suggest that only N-terminal sequence diversity among the SPA proteins could not explain their partial distinct functions in light and dark grown seedlings. To investigate whether distinct *SPA* function are due to differential *SPA* expression *SPA* transcript levels were analyzed under various light regimes and developmental stages. Parts of the *SPA* transcript analyses were conducted during my diploma work and described in my diploma thesis, but are also presented in this work for a complete understanding of *SPA* transcript regulation.

II.2.1. *SPA1* mRNA accumulates in blue light

Previous studies showed that *SPA1* transcript levels are increased in seedlings transferred from darkness to Rc or FRc (Hoecker et al., 1999). Because *spa1* mutants are hypersensitive to Bc, the effect of Bc on *SPA1* transcript levels was determined. To this end, RNA was isolated from dark-grown seedlings as well as seedling transferred to Bc and determined *SPA1* transcript levels by RNA blot analysis.

After two hours of Bc treatment, *SPA1* mRNA accumulated to levels 5- 10-fold higher than in darkness and sustained at high levels after prolonged Bc irradiation (Figure 10 A). *SPA1* transcript levels were not influenced within the first 30 minutes after Bc treatment, but accumulated to high levels after 60 minutes. These results indicate that Bc has a similar influence on *SPA1* mRNA levels as Rc and FRc (Fittinghoff et al., 2006).
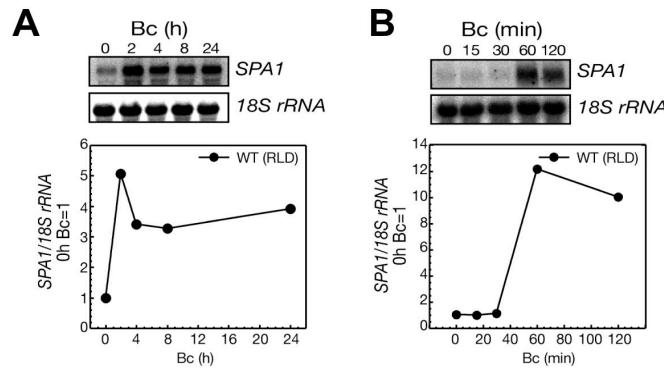
Figure 10: Blue light increases *SPA1* mRNA abundance.

A, B: Total RNA gel blot analysis (top) and quantification (bottom) of *SPA1* transcript levels in seedlings that were transferred from darkness to 5 µmol m^{-2} s^{-1} Bc for 0-24h. Transcript levels were normalized by re-hybridization with an *18SrRNA*-specific probe.

Blue light is perceived by the photoreceptors phyA, cry1 and cry2. phot1 and phot2 are also involved in blue light perception but it was shown that they do not play an important role in B regulation of transcripts (Briggs and Christie, 2002; Briggs and Spudich, 2005). To analyze which photoreceptor is responsible for *SPA1* mRNA accumulation in Bc, *SPA1* transcript levels were analyzed in WT, *phyA*, *cry1 cry2* double and in *phyA cry1 cry2* triple mutant seedlings exposed to low or high fluence rates of Bc.
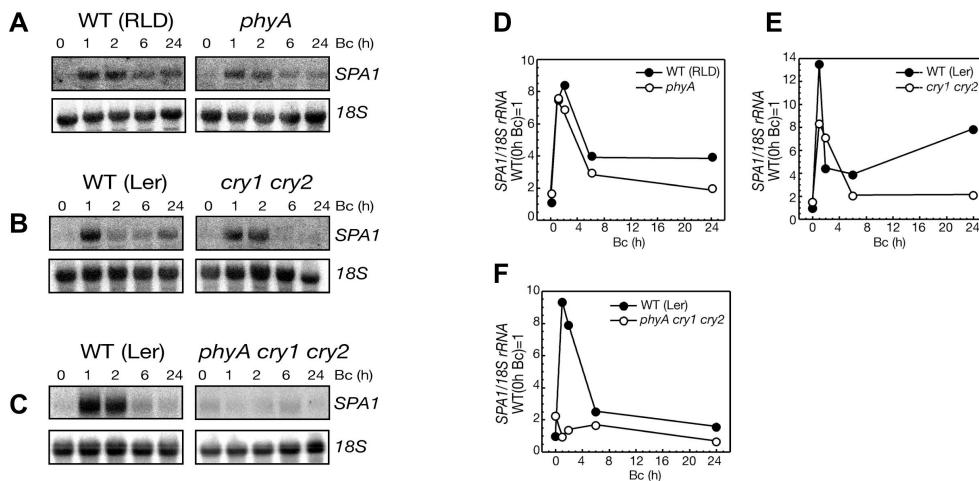
Figure 11: Accumulation of *SPA1* mRNA in high B depends on phyA, cry1 and cry2.

RNA-gel-blot analysis (**A, B, C**) and quantification (**D, E, F**) of *SPA1* transcript levels in *phyA*, *cry1 cry2* and *phyA cry1 cry2* mutant seedlings in comparison to wild-type seedlings (WT: Ler, RLD). Seedlings were transferred from darkness to 5 µmol m^{-2} s^{-1} Bc for 0-24 h. Transcript levels were normalized by re-hybridization with an *18SrRNA*-specific probe.

SPA1 transcript levels in *phyA* mutant seedlings exposed to high fluence rates of Bc light (5 µmol m^{-2} s^{-1} Bc) were similar to those of WT seedlings (Figure 11 A and D). *SPA1* mRNA accumulation in *cry1 cry2* double mutant was somewhat different (Figure 11 B and E). Early accumulation of *SPA1* transcript in *cry1 cry2* double mutant was weaker than in WT, but still detectable, whereas after two hours of Bc exposure the amount of *SPA1* mRNA was strongly reduced when compared to WT (Figure 11 B and E). Only in *phyA cry1 cry2* mutant seedlings, Bc induced accumulation of *SPA1* mRNA was completely abolished (Figure 11 C and F).

phyA mutants irradiated with lower fluence rates of Bc (0.3 µmol m^{-2} s^{-1} Bc) showed reduced amounts of *SPA1* transcript whereas *cry1 cry2* mutant seedlings did not show any differences in *SPA1* mRNA accumulation when compared to WT seedlings (Figure 12). The relevance of cry1 and cry2 for *SPA1* transcript accumulation under low fluence rate of Bc became only obvious in the *phyA cry1 cry2* mutant, in which *SPA1* transcript levels are not responsive to Bc anymore.

Taken together, Bc dependent accumulation of *SPA1* transcript depends on functional phyA, cry1 and cry2. More specifically, cry1 and cry2 play predominant roles in high and phyA major functions in low fluence rates of Bc.
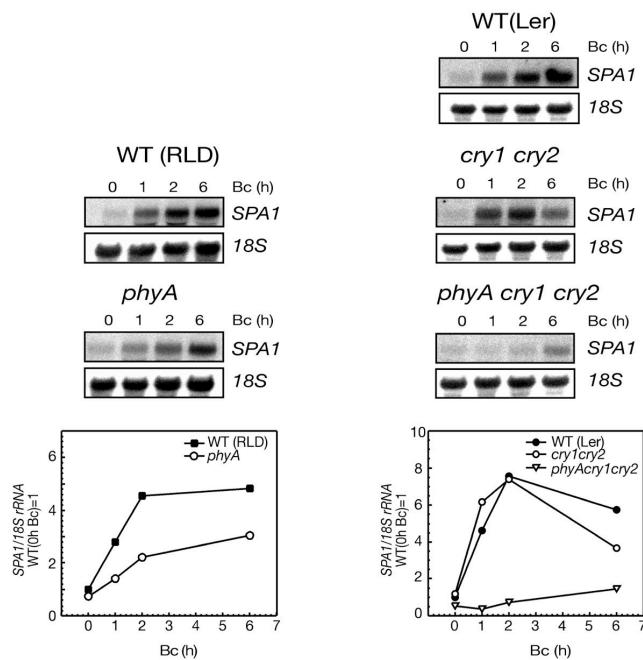
Figure 12: phyA, cry1 and cry2 act redundantly in controlling *SPA1* mRNA levels in low B.

Total RNA-gel-blot analysis (at the top) and quantification (at the bottom) of *SPA1* mRNA from wild-type (RLD/Ler), *phyA* (RLD)*, cry1 cry2* (Ler)*, phyA cry1 cry2* (Ler) mutant seedlings that were transferred from darkness to 0.3 µmol m^{-2} s^{-1} Bc for 0-6 hours. Transcript levels were normalized by re-hybridization with an *18SrRNA*-specific probe.

II.2.2. *SPA3* and *SPA4* but not *SPA2* mRNA levels increase by light

For *SPA1* transcript levels it was already shown that its transcript levels increase in FRc, Rc and Bc (Hoecker et al., 1999, this study). To further analyze if the partial distinct functions of *SPAs* in dark- and light-grown seedlings are based on different light-regulated *SPA* transcript abundance, the transcript levels of *SPA2, SPA3* and *SPA4* were analyzed under different light conditions. To this end, total RNA from dark-grown seedlings as well as seedling transferred to FRc, Rc or Bc were analyzed and *SPA2, SPA3* and *SPA4* transcript levels were determined by RNA blot analysis.

Similar to *SPA1,* the *SPA3* and *SPA4* transcript levels were strongly increased when dark-grown seedlings were transferred to light. Seedlings exposed to either high intensities of Rc, FRc or Bc, respectively, exhibited a 6- to 10-fold increased in *SPA3* or *SPA4* mRNA levels when compared to dark-grown seedlings (Figure 13). The increase of *SPA3* and *SPA4* transcript levels was detectable after two hours of light exposure and stayed at higher levels for all analyzed later time points. In contrast to that, exposure to light did not alter *SPA2* transcript abundance (Figure 13).

Taken together, *SPA1, SPA3* and *SPA4* transcript levels are increased by light, nicely correlating with their function in regulating seedling development in the light. *SPA2* function is mainly restricted to seedling development in darkness, which is in agreement with *SPA2* mRNA levels not being influenced by light.
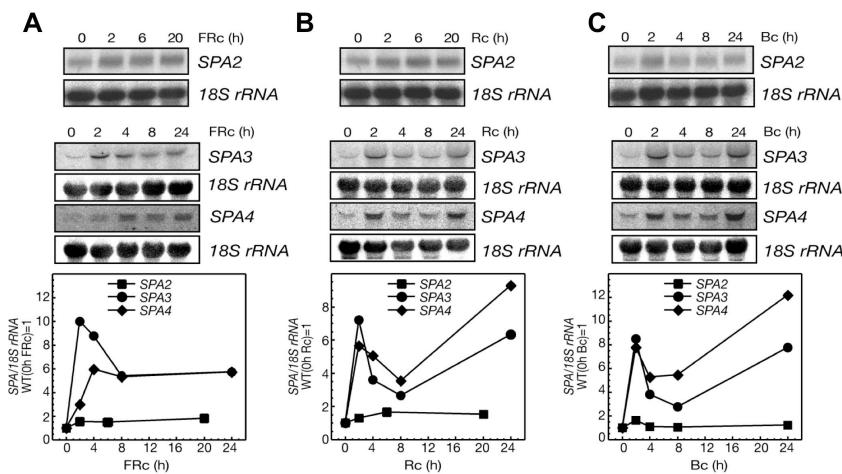
Figure 13: *SPA3* and *SPA4,* but not *SPA2,* transcript levels increase in light.

A, B, C: Total RNA-gel-blot analysis (top) and quantification (bottom) of *SPA2*, *SPA3* and *SPA4* accumulation in 4-day-old dark-grown seedlings (wild type RLD) transferred from darkness to 3 μmol m^{-2} s^{-1} FRc (A) 30 μmol m^{-2} s^{-1} FRc (B) or 5 μmol m^{-2} s^{-1} Bc (C) for 0-24 h. *SPA2*, *SPA3* and *SPA4* signals were normalized to *18SrRNA* levels after phosphoimager quantification.

To uncover which photoreceptors mediate light dependent accumulation of *SPA3* and *SPA4* transcript levels, *SPA3* and *SPA4* transcript levels were determined in WT and in several photoreceptor mutants. After one hour exposure to low intensities of FRc, *SPA3* and *SPA4* transcript levels were strongly induced in the WT. In *phyA* mutants, the increase of *SPA3* and *SPA4* mRNA levels was undetectable (Figure 14). These results are consistent with our knowledge that phyA is the only photoreceptor able to respond to FRc (Casal et al.1997).

Unlike FR, R light signaling depends on functional phyA, phyB, phyC, phyD and phyE whereby phyB plays the predominant role (Reed et al., 1994; Aukerman et al., 1997; Mathews and Sharrock, 1997). In Rc light, *SPA3* and *SPA4* mRNA levels showed an early increase after one hour that was only slightly affected in *phyA* mutants, but completely lost in *phyA phyB* double mutant (Figure 14 B and D).
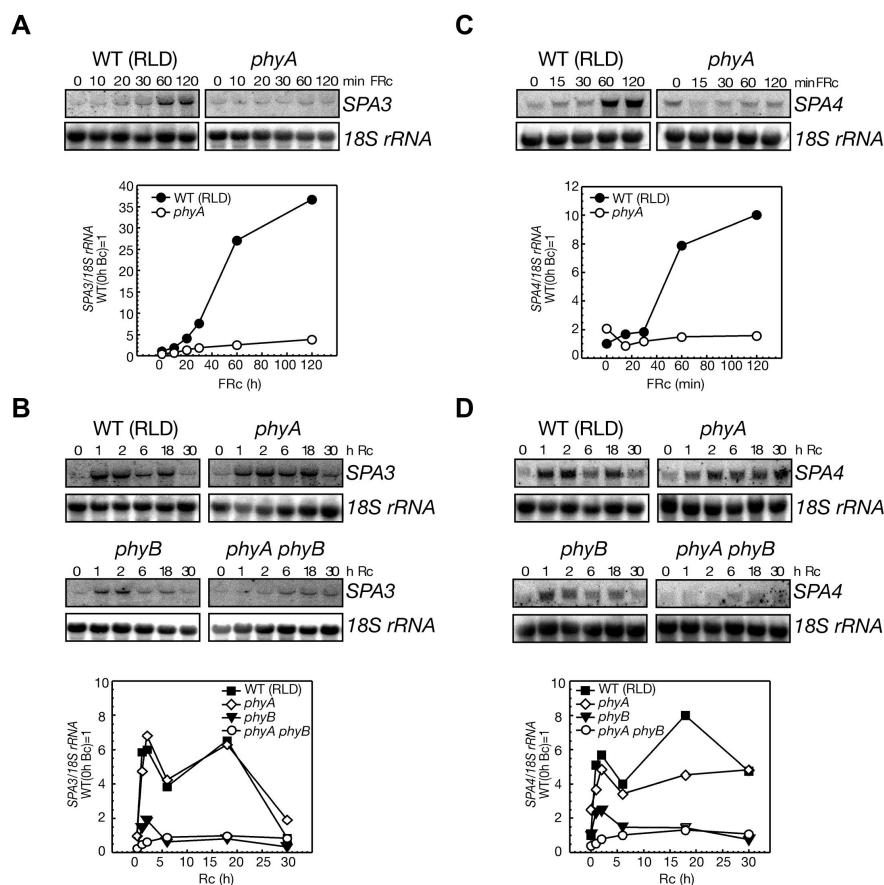
Figure 14: Accumulation of *SPA3* and *SPA4* mRNA in FRc or Rc requires functional phyA or phyB, respectively.

A, B Total RNA gel blot analysis and quantification of *SPA3* (A) and *SPA4* (B) *mRNA* levels from wild-type (RLD) and *phyA* mutant seedlings transferred from darkness to 0.3 μmol m^{-2} s^{-1} FRc for 0-2 h.
C, D Quantification of *SPA3* (C) and *SPA4* (D) transcript accumulation in wild-type (RLD), *phyA*, *phyB*, *phyA phyB* mutant seedlings that were transferred from darkness to 30 μmol m^{-2} s^{-1} Rc for 0-24 h. All blots were reprobed by an *18S rRNA*–specific probe. *SPA3* and *SPA4* signals were normalized to *18S rRNA* levels after phosphorimager quantification.

In low Bc, the increase of *SPA3* and *SPA4* transcript levels was not detectable in phyA and *phyA cry1 cry2* triple mutant. *cry1 cry2* mutants did not show altered regulation of *SPA3* and *SPA4* transcript abundance, indicating that phyA is the photoreceptor mediating increase of *SPA3* and *SPA4* transcript levels in low intensities of Bc. In high fluence rates of Bc, lack of phyA had no effect on *SPA3* and *SPA4* transcript levels. Only in *phyA cry1 cry2* triple mutant seedlings B-light dependent increase in *SPA3* and *SPA4* transcript levels disappeared.

Taken together, *SPA3* and *SPA4* transcript levels increase in all investigated light qualities and show an expression pattern very similar to that of *SPA1*. phyA is responsible for increase of *SPA* levels in FR and low B light, whereas, phyB is mainly involved in the accumulation of *SPA3* and *SPA4* transcripts in Rc. cry1 and cry2 are mainly responsible for increasing *SPA3* and *SPA4* mRNA levels in Bc of high intensities.

II.2.3. *SPA* mRNA abundance partially correlates with its distinct functions during plant development

SPA transcript analysis implies that differences in the regulation of *SPA* expression might contribute to distinct *SPA* functions in dark- and light-grown seedlings. To test whether absolute amounts of *SPA* transcripts correlates with distinct functions in light-, dark-grown seedlings and adult plants, a comparison of *SPA* transcript abundance were performed. While *SPA1* and *SPA2* play predominant roles in light and dark-grown seedling, *SPA3* and *SPA4* mainly regulate vegetative adult plant growth. Therefore, poly(A)+ RNA from seedlings grown for 4 days in darkness or FRc as well as from adult rosettes leaves were isolated and *SPA* transcript levels were subsequently determined by RNA blot analysis. In order to make band intensities of the different *SPA* genes comparable, *SPA* signal were normalized with the respective *UBQ10* signals. This normalized ratio was further corrected for differences in *SPA* probe sizes (see Materials and Methods for details).

In dark-grown seedlings, *SPA1, SPA2, SPA3 and SPA4* transcripts were relatively low abundant. However, *SPA2* is the most abundant *SPA* transcript in dark-grown seedlings (Figure 15). In light grown seedlings, *SPA1*, *SPA3* and *SPA4* transcripts are more abundant than in dark-grown seedlings while *SPA2* transcript levels do not differ between light- and dark-grown seedlings. A direct comparison of *SPA* mRNA levels revealed that *SPA1* and *SPA3* are the most abundant *SPA* transcripts in light

grown seedlings (Figure 15 B). In adult plants, *SPA3* and *SPA4*, but not *SPA2* mRNA levels are strongly increased compared to the seedling stage. Levels of *SPA1* mRNA in adult plants were almost unchanged when compared to the levels in light-grown seedlings. *SPA3* was the most abundant transcript in adult plants. Taken together, comparison of *SPA* transcript levels showed that *SPA2* mRNA levels are largely unaffected among the different developmental stages analyzed. In contrast to that, *SPA1*, *SPA3* and *SPA4* mRNA levels are very low in dark-grown seedlings, higher in light-grown seedlings and reach the maximum in adult plants. These expression patterns partially correlate with the known, distinct *SPA* functions in regulating plant development.
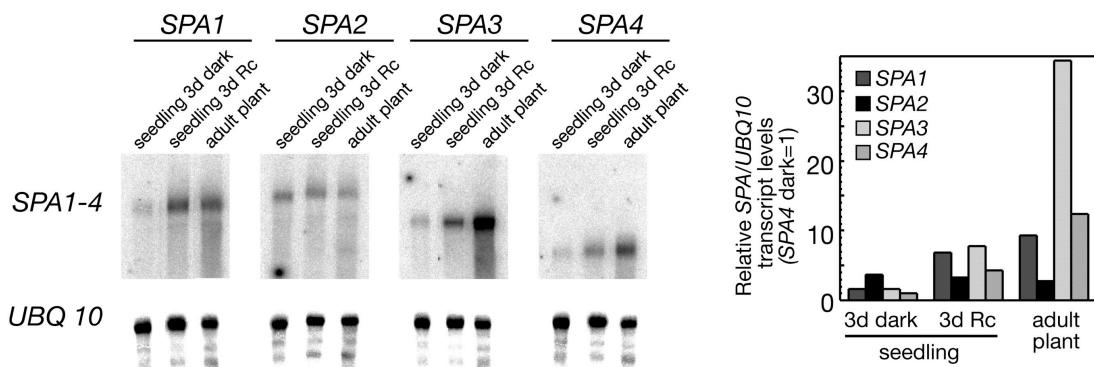
Figure 15: Analysis of *SPA1-SPA4* transcript levels during plant development

A: Comparative *poly(A)*+RNA gel blot analysis of *SPA1*, *SPA2*, *SPA3* and *SPA4* mRNA levels in seedlings grown in darkness or Rc (30 µmol m^{-2} s^{-1}) for 3 days or in 4 week-old adult plants. *SPA* mRNAs were detected with *SPA*-specific probes (for detail see material and methods). For normalization, blots were reprobed with an *UBIQUITIN 10* (*UBQ10*) -specific probe.
B: Quantification of the *SPA* transcript levels shown in A.

II.3. *SPA* Promoter GUS analyses

II.3.1. *SPA1* and *SPA2* but not *SPA4* promoters are strongly active in the roots of young plants

While RNA-blot analysis gains important information about *SPA* mRNA abundance under various light conditions and developmental stages, the weakness of this approach is that especially spatial distribution of *SPA* transcripts within a tissue cannot be resolved. However, this is of utmost importance because some targets of the COP1-SPA complexes like CO are only localized in specialized cell types. To uncover the spatial distribution of *SPA* expression, a promoter-reporter-gene analysis

was conducted with the focus on *SPA1*, *SPA2* and one member of the highly redundant *SPA3*/*SPA4* subgroup, *SPA4*. The reporter gene *GUS* was transformed under the control of either *SPA1*-, *SPA2*- or *SPA4*- 5´regulatory sequences in wild-type plants (Figure 16A). For each construct approximately 70 transgenic T1 plants were analyzed after selection on kanamycin plates. The reason for this high number of transgenic T1 plants is that not only the promoter but also the insertion site can influence the GUS expression pattern. All following results were found in at least 50% of all analyzed lines and therefore likely represent the native SPAX promoter activity. For the analysis of *SPA::GUS* expression in seedlings, at least 20 to 30 independent T2 lines were analyzed.
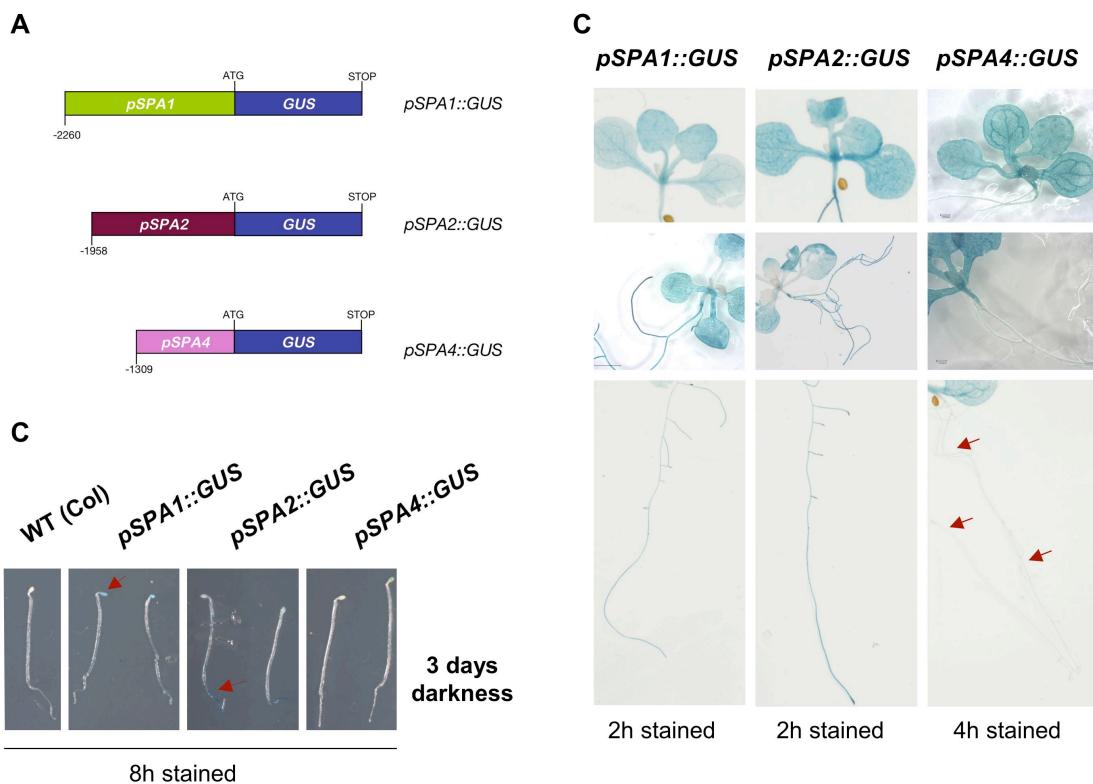
Figure 16: Promoter of *SPA1 and SPA2* are active in roots of seedlings and young plants.

A: Schematic representation of used constructs. The reporter-gene GUS was expressed under 5`regulatory sequences of *SPA1*, *SPA2* or *SPA4.*
B: Visual phenotypes of wild-type (WT) and transgenic T2 plants expressing GUS under the control of *SPA1*, *SPA2* or *SPA4* promoters described in A.
C: Visual phenotypes of segregating transgenic dark-grown wild-type seedlings expressing GUS under control of *SPA1*, *SPA2*, *SPA4* promoters in T1 generation. Plants were grown for two weeks on kanamycin plates.

In dark-grown seedlings, *SPA1* and *SPA2* promoters were predominant active in cotyledons, whereas *pSPA4::GUS* expression was not detectable in dark-grown seedlings (Figure 16). Young plants expressing GUS under the control of either

SPA1 or *SPA2* promoter exhibited GUS staining in roots, hypocotyls and in true leaves (Figure 16). T1 plants carrying the *pSPA4::GUS* transgene had to be stained two times longer than those carrying *SPA1* or *SPA2* promoter, which indicates that the *SPA4* promoter is less active than those of *SPA1* and *SPA2.*

Figure 17: Promoter of *SPA1* and *SPA4* are active in vascular bundles of leaves.

Rosette leaves (first two columns), cauline leaves (third column) and inflorescence (fourth column) of 6 week-old transgenic plants expressing GUS under the control of *SPA1*-, *SPA2*- or *SPA4*- promoter. All plants were selected on kanamycin plates, transferred to soil and grown in LD for three weeks. All tissues were stained at 37°C for 8 hours.

Obvious difference in *SPA* promoter activity was found in the roots. *pSPA1::GUS* and *pSPA2::GUS* reporter constructs were strongly expressed in roots indicated by detectable GUS staining after only a view minutes, whereas GUS activity controlled by *SPA4* promoter was not or only barely detectable even after several hours of staining (Figure 16). Analysis of older rosette leaves showed that promoters of *SPA4* and *SPA1* were strongly active in vascular bundles (Figure 17). In contrast, *SPA2* promoter conferred strong activity in the leaves, but its expression is not restricted to vascular tissues. *SPA* genes are also expressed in cauline leaves and stems (Figure 17). All analyzed *SPA* promoters were active in reproductive tissues. GUS expression was detectable in all flower organs as well as young siliques (Figure 17). Taken together, the *SPA-promoter::GUS* analysis demonstrates that the selected *SPA1* and *SPA2* 5´regulatory regions confer to strong expression in roots of young plants, whereas *SPA4* promoter show no or only weak detectable activity in roots.

Activity of *SPA1* and *SPA4* promoter is strong and spatially restricted to vascular bundles of rosette leaves in adult plants, whereas *SPA2* promoter activity is more widespread in rosettes leaves.

II.4. Promoter-swap analysis

SPA transcript expression analysis and *SPA* promoter GUS experiments revealed that *SPA* genes are differentially regulated depending on light regime, developmental stage and cell type. To answer the question whether differential regulation of *SPA* genes is responsible for their distinct functions, a *SPA* promoter-swap analysis with the focus on *SPA1*, *SPA2* and one member of the highly redundant *SPA3*/*SPA4* subgroup, *SPA4* was designed. Therefore, the cDNAs of *SPA1*, *SPA2* and *SPA4* were expressed either under the control of their endogenous 5' and 3' regulatory sequences or that of *SPA1*. In addition, *SPA1* cDNA was placed under the control of either *SPA2* or *SPA4* 5' and 3' regulatory elements. For simplification, I will name the chosen 5' and 3' regulatory sequences of all *SPAs* promoters. In general, 2 kb upstream of the start codon and approximately 800 bp downstream the stop codon were aspired. Depending on adjacent genes at *SPA* loci and optimized primer annealing for cloning strategy the chosen *SPA* promoters varies in size.

II.4.1. Expression of *SPA1*, *SPA2* and *SPA4* in *spa* triple and quadruple mutants phenocopies appropriate mutant phenotypes in seedlings

First, I tested whether the chosen regulatory 5' and 3' elements are sufficient for proper *SPA* gene expression. To this end, the cDNAs of *SPA1*, *SPA2* and *SPA4* were placed under the control of their endogenous promoters and subsequently introduced into segregating *spa1 spa2 spa3 spa4*/*SPA4* mutants (Figure 18 A). Thus, the resulting T1 generation contains *spa* quadruple mutants as well as homozygous *spa1 spa2 spa3* and heterozygous *spa1 spa2 spa3 spa4/SPA4* triple mutants. It was expected that 1/4 of resulting transgenic T2 plants should be in a quadruple mutant background. Various T2 plants were propagated and an unexpected low yield of *spa* quadruple mutants was obtained (less than 1/10). This might be due to reduced viability or transformability of the *spa1 spa2 spa3 spa4* quadruple mutant embryo or gametes. Because of the low yield of transgenic lines in quadruple background, *spa1 spa2 spa3* triple mutants were also analyzed, because these mutants have similar defects in suppressing photomorphogenesis in the dark and in the light.

In the dark, the vast majority of the analyzed T2 progeny that carries a *pSPA1::cSPA1-HA* or a *pSPA2::cSPA2-HA* construct exhibited, like wild-type seedlings, long hypocotyls and closed cotyledons (Figure 18 B). Thus, these transgenic quadruple mutants were indistinguishable from *spa2 spa3 spa4* or *spa1 spa3 spa4* triple mutants, respectively. These results demonstrate that the chosen regulatory sequences for expression of *SPA1* or *SPA2*, respectively, are sufficient for normal *SPA1* and *SPA2* function.

constructs transformed in spa1 spa2 spa3 spa4 +/-	total* D	spa1 spa2 spa3 spa4 D
pSPA1::cSPA1-HA	35/35	1/1
pSPA2::cSPA2-HA	96/97	6/6
pSPA4::cSPA4-HA	45/52**	3/4
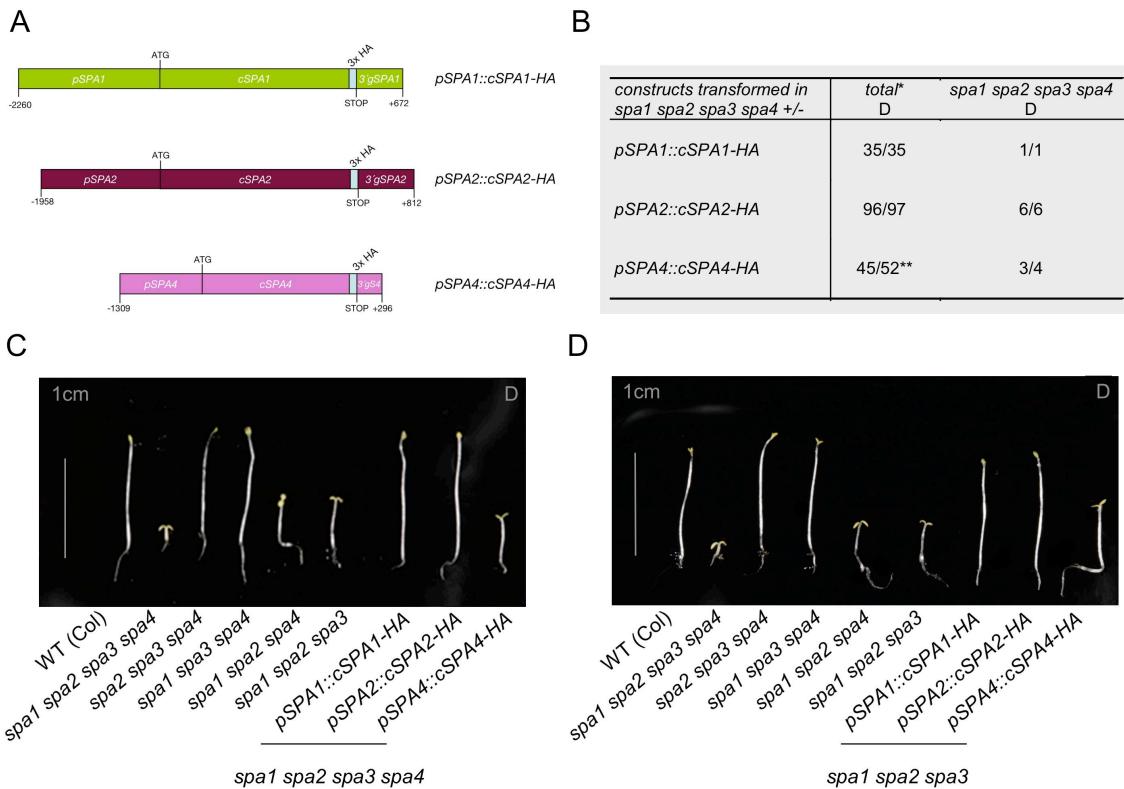
Figure 18: HA tagged *SPA1*, *SPA2* or *SPA4* expressed in *spa* quadruple *or spa1 spa2 spa3* triple mutant are fully functional in the dark.

A: Schematic illustration of *SPA* promoter/cDNA constructs. *SPA1, SPA2* and *SPA4* cDNA were placed under the control of their respective 5' and 3' regulatory sequences.
B: Analysis of T2 seedlings carrying *SPA* promoter/cDNA constructs. *pSPA1::cSPA1-HA*, *pSPA2::cSPA2-HA* and *pSPA4::cSPA4-HA* constructs were transformed into *spa1 spa2 spa3 spa4/SPA4* plants and resulting T2 seedlings were grown for 4 days in complete darkness. The number of individual transgenic lines with long hypocotyls is given. The number of transgenic lines in a *spa quadruple* mutant is presented separately. * Total means all investigated lines in *spa1 spa2 spa3* or *spa1 spa2 spa3 spa4* or spa1 *spa2 spa4 spa4/SPA4 background.* **Denote that *pSPA4::cSPA4-HA* lines show hypocotyls lengths somewhat longer than *spa1 spa2 spa3* triple or quadruple mutants, but still shorter than WT.
C, D: Visual phenotype of dark-grown wild type (WT (Col)), *spa* multiple mutants and *pSPA1::cSPA1-HA*, *pSPA2::cSPA2-HA* and *pSPA4::cSPA4-HA* transgenic lines in a *spa* quadruple (C) or in a *spa1 spa2 spa3* triple (D) mutant background.

SPA4 alone is not able to completely suppress photomorphogenesis in the dark (Laubinger et al., 2004). Consequently, dark-grown *spa* quadruple mutants

expressing *pSPA4::cSPA4-HA* construct exhibited short hypocotyls and expanded cotyledons. However, these seedlings showed hypocotyls longer than that of *spa* quadruple mutants and were indistinguishable from *spa1 spa2 spa3* triple mutants (Figure 18). Hence, *pSPA4::cSPA4-HA* is fully functional and mimics endogenous *SPA4* function.

When *pSPA4::cSPA4-HA* was introduced in *spa1 spa2 spa3* triple mutants, the resulting seedlings exhibited longer hypocotyls than the *spa1 spa2 spa3* triple mutant. These results indicate that an additional copy of a functional *SPA4* gene partially complements the mutant phenotype of *spa1 spa2 spa3* triple mutants.

Taken together, the results show that *pSPA1::cSPA1-HA*, *pSPA2::cSPA2-HA* and *pSPA4::cSPA4-HA* function like their endogenous counter parts in dark-grown seedling. Next, complementation of light-grown transgenic seedlings carrying *pSPA1::cSPA1-HA*, *pSPA2::cSPA2-HA* and *pSPA4::cSPA4-HA* was analyzed. Previous analysis of several multiple mutants revealed that *SPA1*, and to a lower extend SPA4, are important for suppressing photomorphogenesis in FRc (Laubinger et al., 2004; Fittinghoff et al., 2006). In contrast, *SPA2* function is almost irrelevant for inhibition of photomorphogenesis in the light (Laubinger et al., 2004).

Expression of *pSPA1::cSPA1-HA* either in *spa* quadruple mutants or in *spa1 spa2 spa3 triple* mutant completely rescued the mutant phenotypes in FRc indicating that the transgenic SPA1 protein in also functional in the light (Figure 19 A, C and D). Hypocotyls of these seedlings were even slightly longer than that of WT, implying that the *SPA1* rescue construct is slightly more active than the endogenous *SPA1* gene. *spa1 spa2 spa3* triple or *spa* quadruple mutant seedlings expressing *pSPA2::SPA2-HA* did not show rescue of the mutant phenotypes and exhibited strong photomorphogenesis with fully opened cotyledons and short hypocotyls in FRc (Figure 19 B - D). This indicates that the transgenic *SPA2* construct, as endogenous *SPA2*, is not able to suppress photomorphogenesis in the light.

Expression of *pSPA4::SPA4-HA* in *spa* quadruple mutants mimicked the phenotype of *spa1 spa2 spa3* triple mutants (Figure 19 B - D). Again, an extra copy of *SPA4* in *spa1 spa2 spa3* triple mutant seedlings resulted in seedlings with longer hypocotyls than the triple mutant (Figure 19 C and D). This probably indicates that low *SPA4* levels are a limiting step in suppressing photomorphogenesis in *spa1 spa2 spa3* triple mutants.

Taken together, expression of *pSPA1::cSPA1-HA* complement *spa quadruple* and *triple* mutant phenotype in darkness and in FRc. As expected fusion of *SPA2-HA* expressed under the control of its own *SPA2* promoter also complement mutant phenotype in darkness, but not in FRc. Transgenic *spa* quadruple mutants carrying *pSPA4::cSPA4-HA* copied the phenotype of *spa1 spa2 spa3* mutant in darkness and FRc.
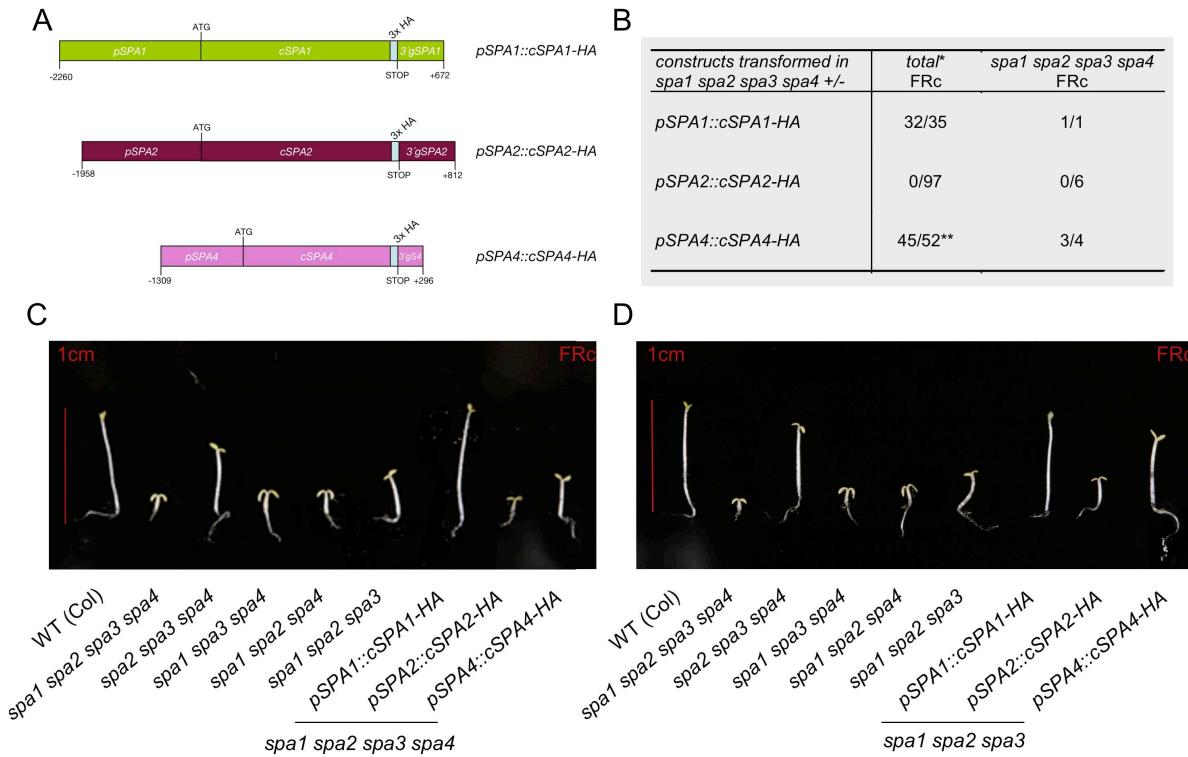
Figure 19: HA tagged *SPA1*, *SPA2* or *SPA4* expressed in *spa* quadruple *or spa1 spa2 spa3* copy its respective mutant phenotypes in light.

A: Schematic illustration of *SPA* promoter/cDNA constructs. *SPA1*, *SPA2* and *SPA4* cDNAs were placed under the control of their respective 5' and 3' regulatory sequences.
B: Analysis of T2 seedlings carrying *SPA* promoter/cDNA constructs. *pSPA1::cSPA1-HA*, *pSPA2::cSPA2-HA* and *pSPA4::cSPA4-HA* constructs were transformed in *spa1*, *spa2*, *spa3*, *spa4/SPA4* plants and resulting T2 seedlings were grown for one day in complete darkness and for further three days in low FRc (0,3 µmol m^{-2} s^{-1}). The number of individual transgenic lines with long hypocotyls is given. The number of transgenic lines in a *spa quadruple* mutant is presented separately. Total means all investigated lines including non-segregating spa quadruple, non-segregating *spa1 spa2 spa3* triple mutants and segregating *spa1 spa2 spa3 spa4/SPA4*. **Denote that *pSPA4::cSPA4-HA* lines show hypocotyls lengths somewhat longer than *spa1 spa2 spa3* triple mutants, but are still shorter than WT.
C, D: Visual phenotype various FR-grown *spa* multiple mutants and *pSPA1::cSPA1-HA*, *pSPA2::cSPA2-HA* and *pSPA4::cSPA4-HA* transgenic lines in a *spa* quadruple (C) or in a *spa1 spa2 spa3* triple (D) mutant background.

II.4.2. In darkness *cSPA2* and *cSPA4* expressed by the *SPA1* promoter complement mutant phenotype

Previous genetic studies revealed that either functional *SPA1* or *SPA2* are sufficient to inhibit photomorphogenesis in darkness, thus *spa* triple mutants carrying either functional *SPA1* or *SPA2* gene, respectively, exhibit long hypocotyls and closed cotyledons like WT seedlings (Laubinger et al., 2004). Therefore, *SPA1* and *SPA2* have very similar functions in dark-grown seedlings. This idea is corroborated by the finding that *spa* quadruple or triple mutants expressing *cSPA2* under the control of the *SPA1* promoter showed normal skotomorphogenesis in darkness like WT (Figure 20).
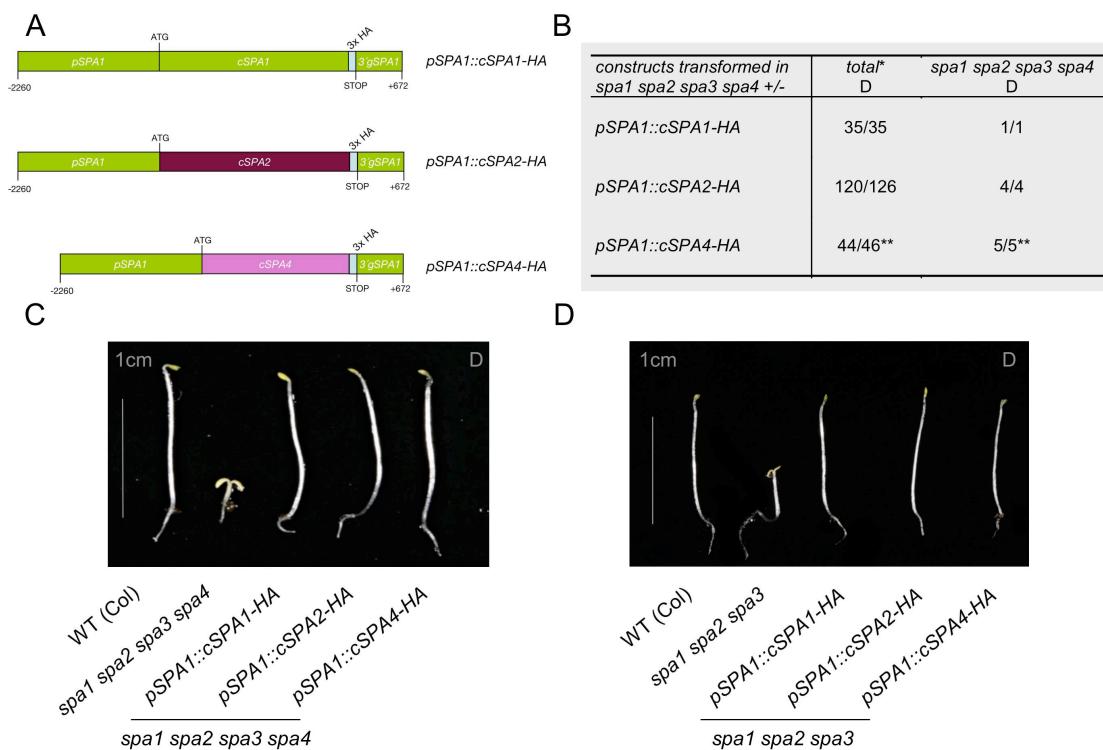
Figure 20: Expression of various *cSPA-HA* fusions by the *SPA1* promoter complement *spa* mutants in darkness.

A: Schematic illustration of *SPA* promoter/cDNA constructs. *SPA1, SPA2* and *SPA4* cDNA were placed under the control of *SPA1* 5' and 3' regulatory sequences.
B: Analysis of T2 seedlings carrying *SPA* promoter/cDNA constructs. *pSPA1::cSPA1-HA*, *pSPA1::cSPA2-HA* and *pSPA1::cSPA4-HA* constructs were transformed in *spa1*, *spa2*, *spa3*, *spa4/SPA4* plants and resulting T2 seedlings were grown for 4 days in complete darkness. The number of individual transgenic lines with long hypocotyls is given. The number of transgenic lines in a *spa quadruple* mutant is presented separately.
C, D: Visual phenotype various dark-grown *spa* multiple mutants and *pSPA1::cSPA1-HA*, *pSPA1::cSPA2-HA* and *pSPA1::cSPA4-HA* transgenic lines in a *spa* quadruple (C) or in a *spa1 spa2 spa3* triple (D) mutant background.

SPA4, like *SPA3*, has only limited functions in suppressing photomorphogenesis in darkness. Hence, in darkness *spa* triple mutant seedlings with either functional *SPA3* or *SPA4* exhibit constitutive photomorphogenesis with only slightly longer hypocotyls than *spa* quadruple seedlings. Interestingly, expression of *cSPA4-HA* fusion under the *SPA1* promoter in a *spa* quadruple mutant was fully able to complement mutant phenotype in darkness (Figure 20). From 46 investigated transgenic lines 44 showed long hypocotyls and closed cotyledons in darkness similar to WT. In contrast, *SPA4* driven by its own promoter, as it is the case in *spa1 spa2 spa3* triple mutants, cannot completely suppress photomorphogenesis in the dark (Figure 18 C and D). Nevertheless, expression of *cSPA4-HA* under control of the *SPA1* promoter demonstrates that the SPA4 protein has the potential to completely suppress photomorphogenesis in the dark

In conclusion, these results revealed that *SPA* gene regulation by *SPA1* promoter is sufficient to inhibit photomorphogenesis in darkness and more relevant than *SPA* protein sequences.

II.4.3. *SPA2* expressed under the control of *SPA1* promoter is not able to rescue mutant phenotypes in FR, whereas *SPA4* does partially

As shown above, *SPA2* expressed under the control of the *SPA1* promoter is sufficient to repress photomorphogenesis in the dark (Figure 21 C and D). These results are not unexpected because both *SPA1* and *SPA2* play very similar roles in dark-grown seedlings. However, *SPA2* function is limited to dark-grown seedlings, while *SPA1* also plays important functions in the light. This might be due to the fact that *SPA1*, but not *SPA2*, transcript levels are increased by light. Consequently, I asked the question whether *SPA2*, when driven by the *SPA1* promoter, is also able to suppress photomorphogenesis in the light.

spa quadruple mutants like *spa1 spa2 spa3* triple mutants expressing *pSPA1::cSPA2-HA* were indistinguishable from *spa* quadruple or triple mutant progenitors (Figure 21 B). These results indicate that the *SPA2* cDNA, even when controlled by the light-inducible *SPA1* promoter, is not functional in the light. In contrast to that, seedlings expressing *SPA4* driven by the *SPA1* promoter in *spa1 spa2 spa3* triple or *spa* quadruple mutants exhibited longer hypocotyls than the respective mutants (Figure 21 C and D).
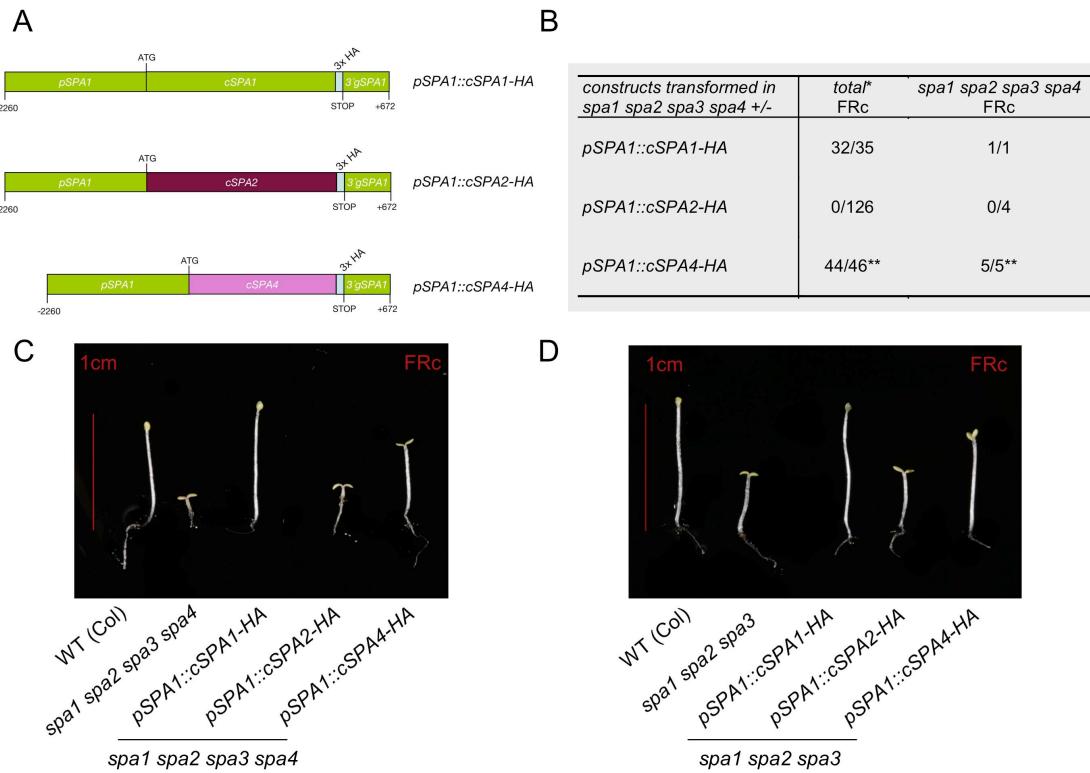
Figure 21: *cSPA2* expressed by *pSPA1* is not functional in the light, whereas *pSPA1::SPA4-HA* is partially active.

A: Schematic illustration of *SPA* promoter/cDNA constructs. *SPA1, SPA2* and *SPA4* cDNA were placed under the control of *SPA1* 5' and 3' regulatory sequences.

B: Analysis of T2 seedlings carrying *SPA* promoter/cDNA constructs. *pSPA1::cSPA1-HA*, *pSPA1::cSPA2-HA* and *pSPA1::cSPA4-HA* constructs were transformed in *spa1 spa2 spa3 spa4/SPA4* plants and resulting T2 seedlings were grown for one day in darkness an further three days in FRc . The number of individual transgenic lines with long hypocotyls is given. The number of transgenic lines in a *spa quadruple* mutant is presented separately.

C, D: Visual phenotypes of various FR-grown *spa* multiple mutants and *pSPA1::cSPA1-HA*, *pSPA1::cSPA2-HA* and *pSPA1::cSPA4-HA* transgenic lines in a *spa* quadruple (C) or in a *spa1 spa2 spa3* triple (D) mutant background grown in low fluence rates of FR (0.3 µmol m^{-2} s^{-1})

To further show that *SPA4* expressed under *SPA1* promoter is more active than under its own promoter *pSPA1::cSPA1-HA, pSPA4::cSPA4-HA, and pSPA1::cSPA4-HA* constructs were transformed into *spa3 spa4* double mutants. *spa3 spa4* double mutants show enhanced photomorphogenesis in the light, but are indistinguishable from WT in the dark (Laubinger and Hoecker, 2003). *spa3 spa4* carrying the *pSPA1::cSPA4-HA* transgene showed much longer hypocotyls than *spa3 spa4* mutant and even longer hypocotyls than WT. In contrast, *spa3 spa4* mutant seedlings transformed with the *pSPA4::cSPA4-HA* construct exhibited hypocotyls lengths similar to that of *spa3* mutants (Figure 22).

A

B

C

constructs transformed in *spa3 spa4*	Rc (rescued/ total)	FRc (rescued/ total)
pSPA4::cSPA4-HA	15/27	15/27
pSPA1::cSPA4-HA	51/55*	51/55*
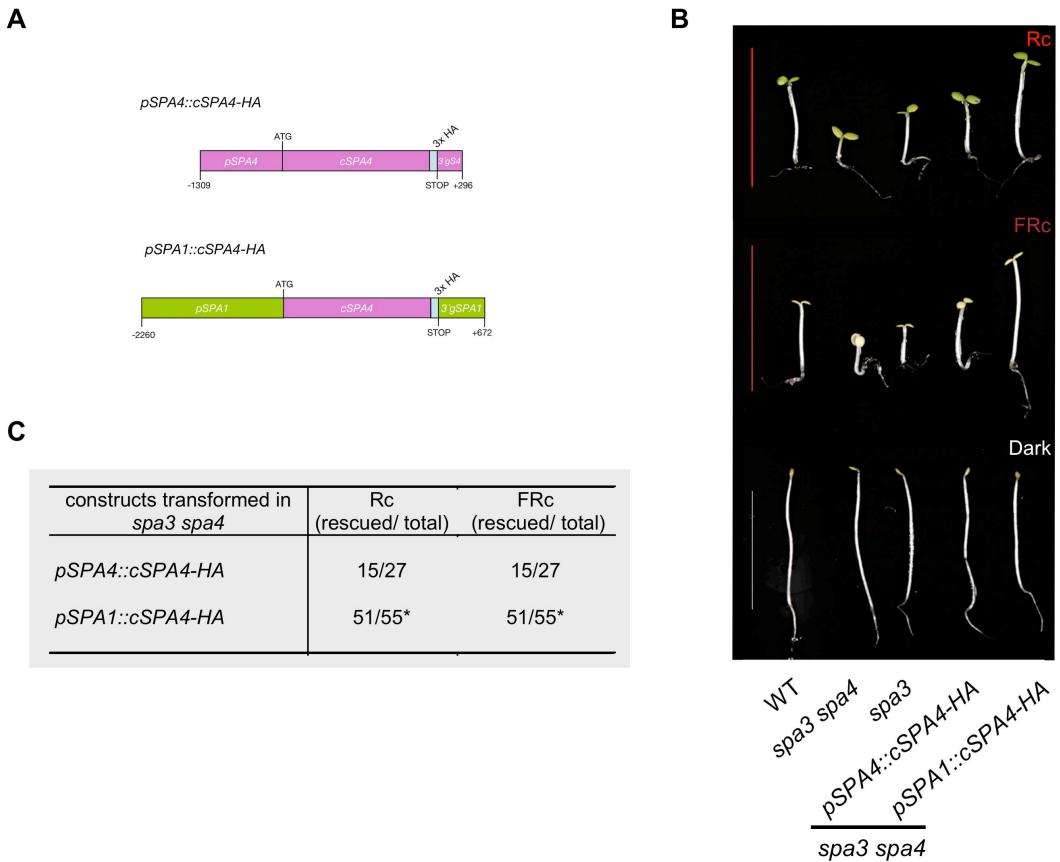
Figure 22: *SPA4 expressed by the SPA1 promoter is more active than expressed under its own regulatory sequences.*

A: Schematic illustration of cSPA4-HA expressed either under its endogenous 5' and 3' regulatory sequences or that of *SPA1*.

B: Visual phenotype of Rc- (top), FRc (middle) or dark-grown (bottom) *spa3 spa4* double, *spa3* single mutants and *pSPA4::cSPA4-HA or pSPA1::cSPA4-HA* transgenic lines in *spa3 spa4* mutant background.

C: Analysis of T2 seedlings carrying *SPA* promoter/cDNA constructs. *pSPA1::cSPA4-HA*, *pSPA4::cSPA4-HA* constructs were transformed in *spa3 spa4* double mutant plants and resulting T2 seedlings were grown for either 4 days in complete darkness or for one day in darkness and further 3 days in FRc or Rc. The number of individual transgenic lines with long hypocotyls is given.

Taken together, it is not sufficient to express the *SPA2* gene under the control of the *SPA1* promoter to mimic the specific function of *SPA1* in the light. These results highlight the function of the *SPA1* cDNA and, most likely, the resulting protein, as a more potent repressor of light signaling when compared to *SPA2*. In contrast, *SPA4*, which acts already as repressors in the light, is much more active under control of the *SPA1* promoter.

II.4.4. *SPA1* under control of *SPA2* promoter rescue mutant phenotypes in darkness and in FR

The results presented in this thesis imply that the SPA1 and SPA4 protein have some intrinsic properties making them potent repressors in light-grown seedlings. In contrast, the SPA2 protein is only functional in the dark, even when expressed from the light-inducible *SPA1* promoter. To test the hypothesis that *SPA1* cDNA already confers properties for a potent repressor in the light, the *SPA1* cDNA was placed under the control of the *SPA2* promoter, the expression of which is not regulated by light.
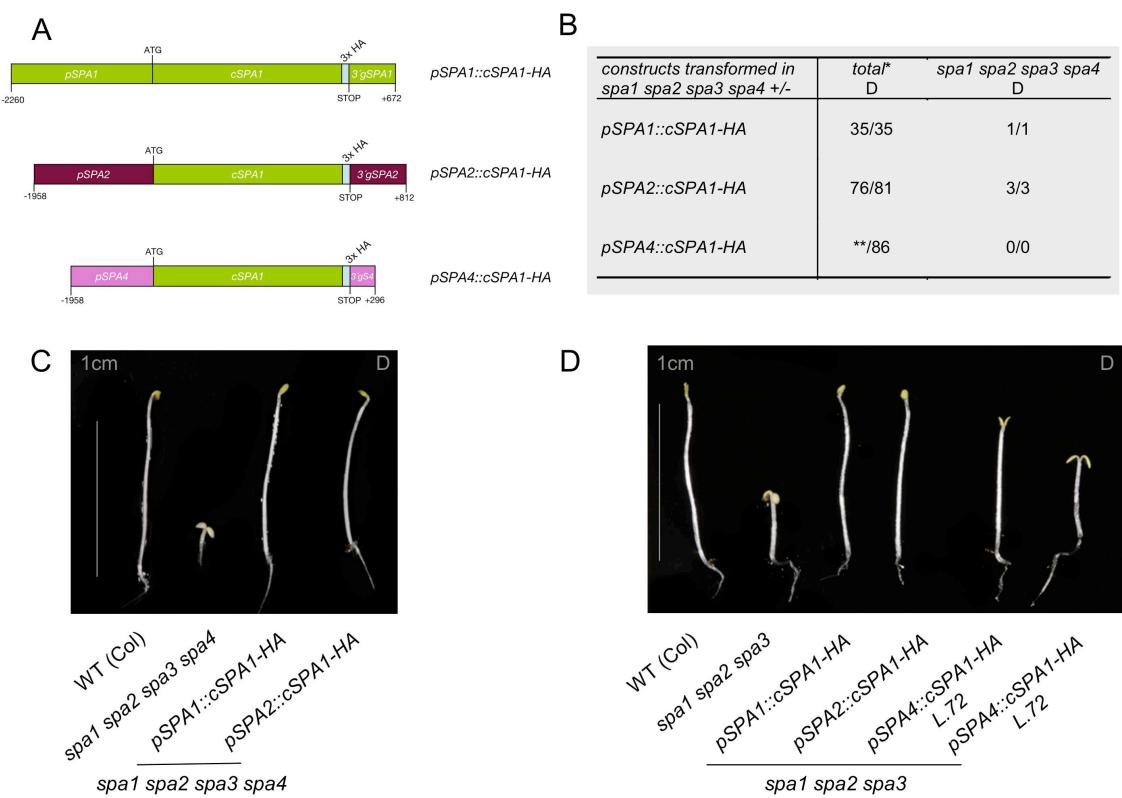
Figure 23: *SPA1* under control of *SPA2* promoter rescues *spa* mutant phenotypes in darkness.
A: Schematic illustration of *SPA* promoter/cDNA constructs. *SPA1* cDNA was placed under the control of *SPA1*, *SPA2* or *SPA4* 5' and 3' regulatory sequences.
B: Analysis of T2 seedlings carrying *SPA* promoter/cDNA constructs. *pSPA1::cSPA1-HA*, *pSPA2::cSPA1-HA* and *pSPA4::cSPA1-HA* constructs were transformed in *spa1 spa2 spa3 spa4*/*SPA4* plants and resulting T2 seedlings were grown for 4 days in complete darkness. The number of individual transgenic lines with long hypocotyls is given. The number of transgenic lines in a *spa quadruple* mutant is presented separately. **Denote that *pSPA4::cSPA1-HA* lines are not analyzable by phenotype.
C, D: Visual phenotype various dark-grown *spa* multiple mutants and *pSPA1::cSPA1-HA*, *pSPA2::cSPA1-HA* and *pSPA4::cSPA1-HA* transgenic lines in a *spa* quadruple (C) or in a *spa1 spa2 spa3* triple (D) mutant background. For *pSPA4::cSPA1-HA* construct, segregating line 72 is presented that exhibits a variety of hypocotyl length.

Dark-grown *spa* quadruple and *spa1 spa2 spa3* triple mutant seedlings expressing *cSPA1-HA* driven by *SPA2* promoter showed long hypocotyls and closed cotyledons similar to WT (Figure 23 C and D). These results are not unexpected, because both SPA1 and SPA2 are sufficient to repress photomorphogenesis in the dark. More interestingly, under FRc conditions transgenic mutants carrying *pSPA2::cSPA1-HA* also rescued the *spa* quadruple or triple mutant phenotype, respectively (Figure 24 C and D). Transgenic seedlings expressing *pSPA1::cSPA1-HA* or *pSPA2::cSPA1-HA* in *spa* quadruple or triple were indistinguishable from WT indicating that SPA1 can fulfil its function in the light even when expressed under the non-light-inducible *SPA2* promoter (Figure 19 C and D).
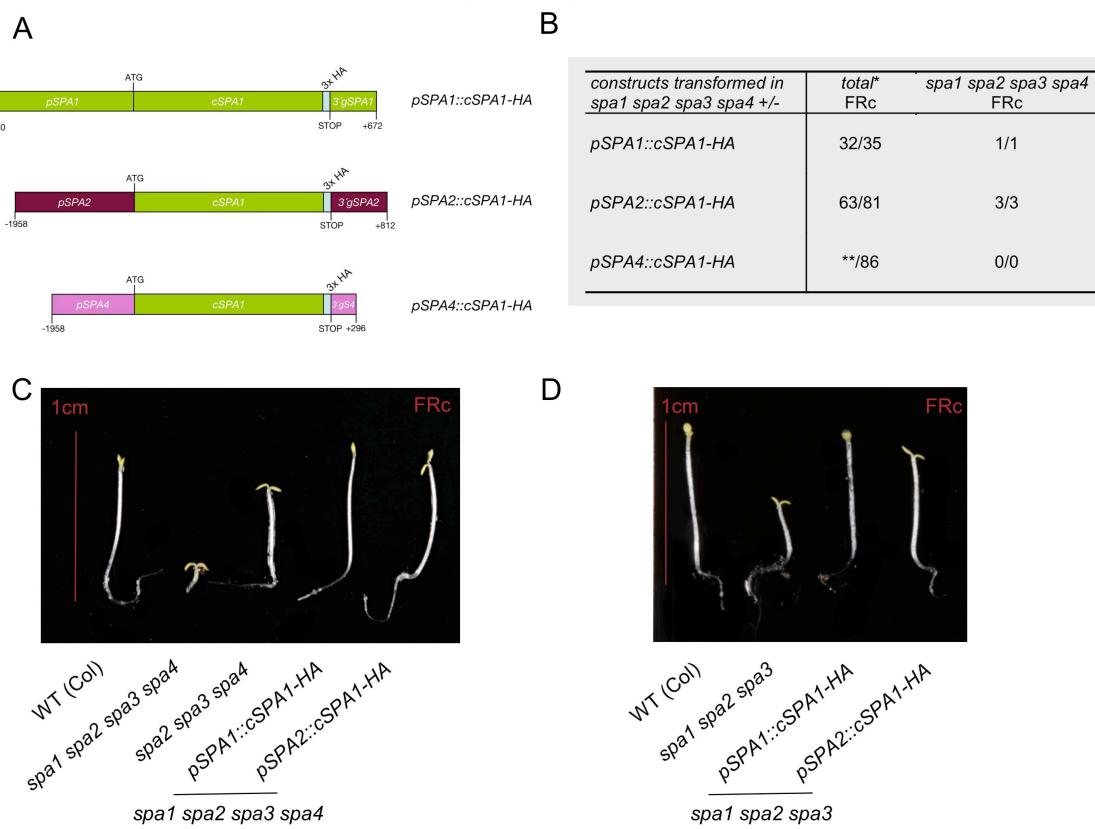

Figure 24: *SPA1* under control of *SPA2* promoter rescues *spa* mutant phenotypes in FR.

A: Schematic illustration of *SPA* promoter/cDNA constructs. *SPA1* cDNA were placed under the control of *SPA1*, *SPA2* or *SPA4* 5' and 3' regulatory sequences.
B: Analysis of T2 seedlings carrying *SPA* promoter/cDNA constructs. *pSPA1::cSPA1-HA*, *pSPA2::cSPA1-HA* and *pSPA4::cSPA1-HA* constructs were transformed in *spa1*, *spa2*, *spa3*, *spa4/SPA4* plants and resulting T2 seedlings were grown for 4 days in complete darkness. The number of individual transgenic lines with long hypocotyls is given. The number of transgenic lines in a *spa quadruple* mutant is presented separately. ** Denote that *pSPA4::cSPA1-HA* lines are not analyzable by phenotype
C, D: Visual phenotype various dark-grown *spa* multiple mutants and *pSPA1::cSPA1-HA*, *pSPA2::cSPA1-HA* and *pSPA4::cSPA1-HA* transgenic lines in a *spa* quadruple (C) or in a *spa1 spa2 spa3* triple (D) mutant background

Analysis of transgenic *spa1 spa2 spa3* mutant seedlings carrying *SPA1* cDNA expressed under the control of the weak *SPA4* promoter turned out to be very difficult. Unfortunately, no single transgenic *spa* quadruple mutant line expressing *pSPA4::cSPA1-HA* out of 86 investigated lines were recovered. Hence, analysis focused on *spa1 spa2 spa3* triple mutants expressing *SPA1* under the control of the *SPA4* promoter. In the dark, none of the segregating T2 lines complemented to WT phenotype. However, *spa1 spa2 spa3* triple mutants expressing *pSPA4::cSPA1-HA* had somewhat longer hypocotyls than the respective mutant. To verify this result, a co-segregation analysis should clear if the seedlings with longer hypocotyls carrying the transgene. Therefore co-segregation analysis was performed with three independent T2 lines segregating 3:1 for the transgene insertion. All lines were grown for four days in complete darkness. From each line, seedlings with the longest or the shortest hypocotyls were selected and genomic DNA was isolated from each single seedling. Afterwards genomic DNA of each seedling was used for PCR with transgene-specific and control oligonucleotides (IV.1.4.).

Due to isolation of genomic DNA out of one single seedling was less efficient, co-segregation results were partially ambiguous. But none of the short dark-grown seedlings did carry the *pSPA4::cSPA1-HA* transgene, while eight out of twelve long dark-grown seedlings were positively tested for the *pSPA4::cSPA1-HA* transgene. However, these results indicate small evidences that expression of *cSPA1* might partially suppress photomorphogenesis in dark-grown seedling when expressed under the control of *SPA4* promoter. Because *SPA1* alone can completely suppress photomorphogenesis in the dark when expressed under *SPA1* or *SPA2* promoter, these results suggest that *SPA4* promoter is too weak to produce sufficient amounts of *SPA1*.

A

pS4cS1

ATG

| pSPA4 | cSPA1 | 3x HA | 3'gS4 |

pSPA4::cSPA1-HA

-1958   STOP +296

B

	amount of seedlings giving pcr product/ total amount of seedlings
long seedlings	8/12
short seedlings	0/12

C

pSPA4::cSPA1-HA #19

D

pS4cS1

MSA6

#19-1 #19-2 #19-3 #19-4 #19-5 #19-6 #19-7 #19-8

spa1 spa2 spa3

D

pSPA4::cSPA1-HA #42

D

pS4cS1

MSA6

#42-1 #42-2 #42-3 #42-4 #42-5 #42-6 #42-7 #42-8

spa1 spa2 spa3

E

pSPA4::cSPA1-HA #72

D

pS4cS1

MSA6

#72-1 #72-2 #72-3 #72-4 #72-5 #72-6 #72-7 #72-8

spa1 spa2 spa3

F

negative controls

positive controls

pS4cS1

MSA6

Figure 25: Co-segregation analysis of dark-grown transgenic *spa1 spa2 spa3* seedlings carrying *pSPA4::cSPA1-HA*.

A: Schematic illustration of *cSPA1* placed under the control of *SPA1* promoter.
B: Summarized illustration of co-segregation results based on PCR amplification.
C, D, E: Visual phenotypes of three independent *spa1 spa2 spa3* triple mutant seedlings probably expressing *SPA4::cSPA1-HA* grown for 4 days in complete darkness (line #19, line#42, line#72) and negative images of PCR products either amplified the transgene (*pS4cS1*) or a region of WT (Col) genomic DNA (*MSA6*).
F: Negative PCR images of negatives and positives controls for correct PCR amplification. As negative controls for *pS4cS1* PCR genomic DNA of *spa1 spa2 spa3* spa4/*SPA4* was used. For the positive control 10 pg of plasmid DNA (*pSPA4::cSPA4-HA* pJHA212-hpt) was used instead of genomic DNA. As negative control for *MSA6* amplification water instead of genomic DNA was used. For positive control of *MSA6* PCR genomic DNA of WT (Col) was amplified.

II.4.5. Promoter-swap analyses in *spa1* single mutants demonstrate that *cSPA1* expressed by *SPA4* is partially functional

Because it is very difficult to analyze the function of *SPA1* driven by the *SPA4* promoter in light-grown *spa1 spa2 spa3* seedlings, the *pSPA4::cSPA1-HA* transgene was also introduced into *spa1* single mutants.

spa1-100 exhibits enhanced de-etiolation response in low FR but is indistinguishable from WT in complete darkness. This makes it easy to analyze the functional potential of SPA1 in the light when driven by the *SPA4* promoter. 25 from 26 transgenic *pSPA4::cSPA1-HA spa1-100* T2 lines showed partial complementation of the *spa1*

mutant phenotype. The rescue was not complete, probably indicating again that the *SPA4* promoter is not strong enough to produce an adequate amount of *SPA1* mRNA. This is again supported by the observation that *SPA1* driven by the *SPA2* promoter completely rescues the phenotype of a *spa1* mutant.

pSPA1::cSPA1-HA, *pSPA1::cSPA2-HA*, *pSPA4::cSPA4-HA* constructs were also introduced into *spa1* mutants. Similar to the results observed in the *spa* quadruple mutant, *SPA4*, but not *SPA1*, was able to mimic the function of *SPA1* in the light. In contrast, an additional copy of the *SPA4* gene in the *spa1* mutant background (*pSPA4::cSPA4-HA spa1*) had no effect on the hypocotyl length.

Taken together, these results further indicate, that *SPA4* has the potential to act as a strong *SPA* repressor in the light, but only when placed under the control of the *SPA1* promoter. In contrast to that, *SPA2* can never act as a repressor in the light, even when expressed under the *SPA1* promoter.

constructs transformed in *spa1-100*	FRc
pSPA1::cSPA1-HA	45/47
pSPA2::cSPA2-HA	0/65
pSPA4::cSPA4-HA	0/8
pSPA1::cSPA2-HA	0/40
pSPA1::cSPA4-HA	25/26
pSPA2::cSPA1-HA	16/20
pSPA4::cSPA1-HA	partial complementation
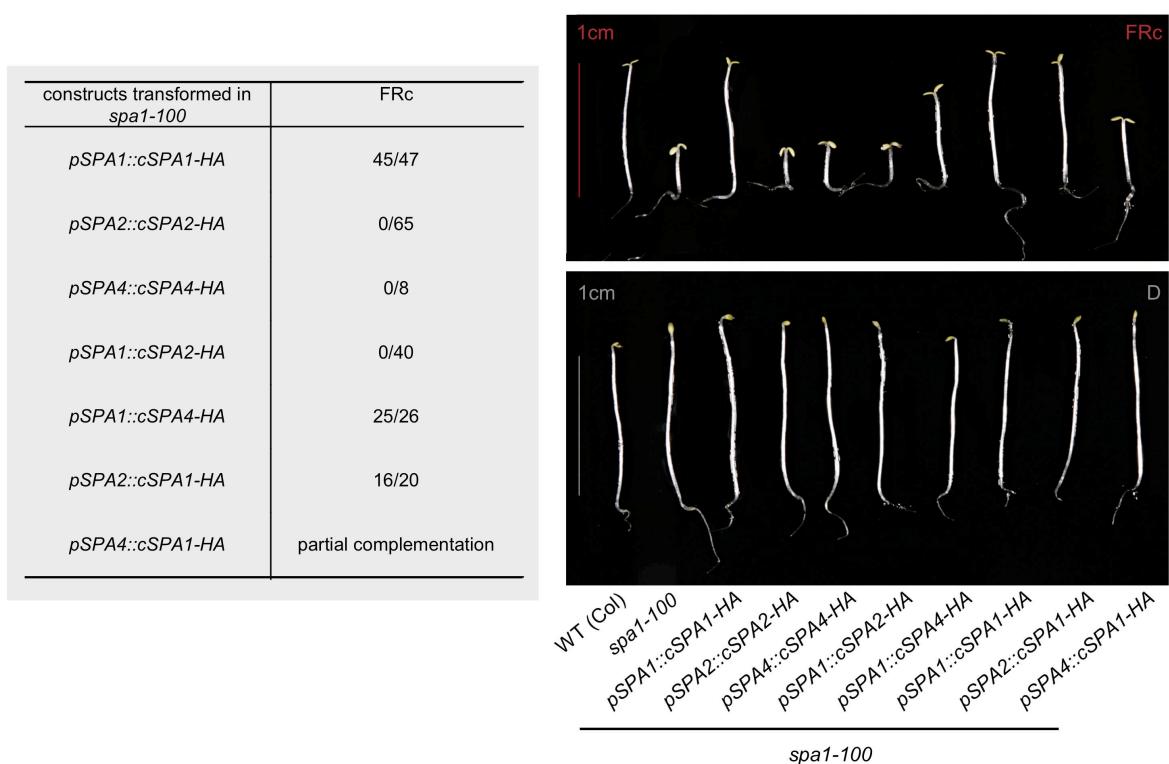
Figure 26: Various *SPA*-promoter-swap constructs expressed in *spa1-100*.

A: Analysis of T2 seedlings carrying all *SPA* promoter/cDNA constructs. *SPA1, SPA2* and *SPA4* cDNA were placed under the control of their respective 5' and 3' regulatory sequence. *SPA2* and *SPA4* were placed under control of *SPA1* 5' and 3' regulatory sequences. *SPA1* cDNA was placed under the control of *SPA2* or *SPA4* promoter. All cDNAs were fused to a triplicate of an HA encoding sequence. All constructs were transformed in *spa1-100* mutant (Col) background. Resulting T2 seedlings were grown for one day in darkness and for further three days in 0,3 µmol m^{-2} s^{-1} FRc.
The number of individual transgenic *spa1-100* lines with long hypocotyls is given.
B: Visual phenotypes of dark- and FR- grown wild-type (WT (Col)), *spa1-100* or transgenic *spa1-100* mutants carrying promoter-swap constructs described in A. Seedlings either grown for four days darkness or one day in darkness and for further three days in 0,3 µmol m^{-2} s^{-1} FR.

39

II.4.6. GUS analyses verified expression of *cGUS-cSPA2-HA* under control of *SPA1* promoter

Above presented results of promoter-swap analysis revealed that the cDNA of *SPA2* is able to rescue the *spa* mutant seedling phenotype in darkness but not in FRc. One possibility why the *SPA2* cDNA does not function in the light is that the SPA2 protein is not expressed or extremely instable in the light. To revise this possibility, the coding sequence of β-glucuronidase (GUS) was fused to respective *SPA* cDNAs. The resulting constructs (*pSPA1::GUS-cSPA1-HA*, *pSPA1::GUS-cSPA2-HA* and *pSPA2::GUS-cSPA2-HA*) were transformed into segregating *spa1 spa2 spa3 spa4/SPA4* (Figure 27 A).
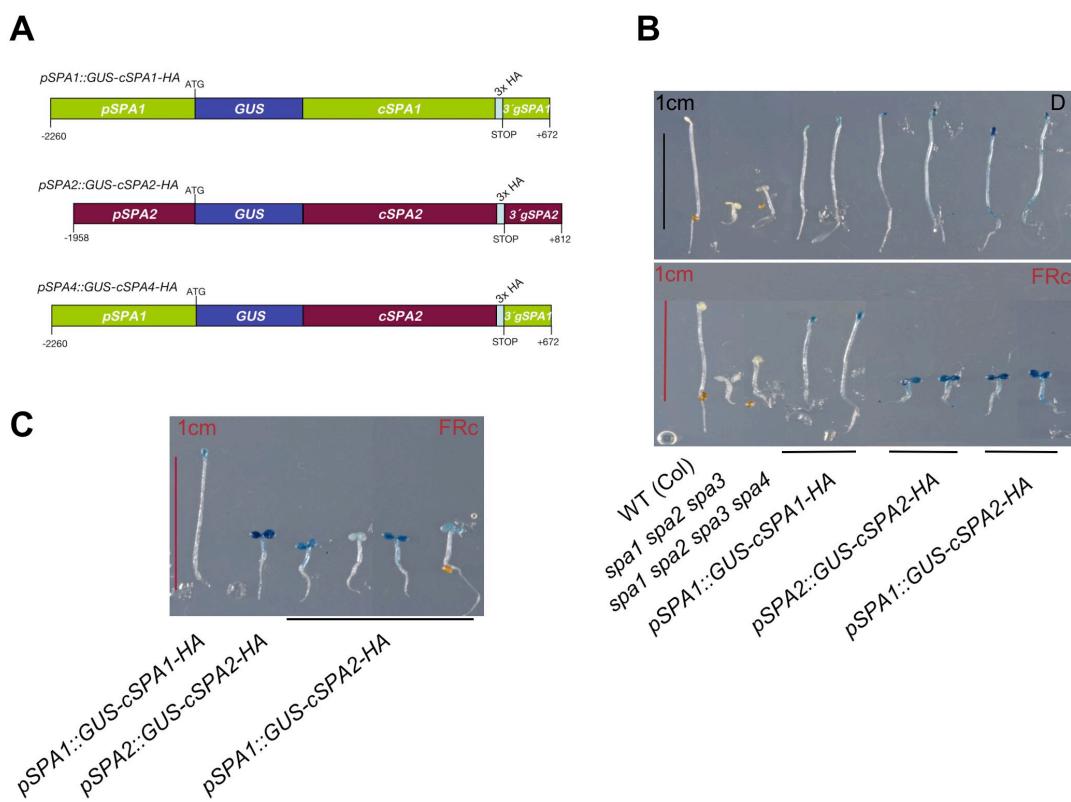
Figure 27: GUS fusion revealed GUS-SPA1-HA or GUS-SPA2-HA expression in transgenic lines.

All shown seedlings are in segregating T2 generation. Those seedlings were grown either for four days in complete darkness or for one day in darkness and for further three days in FR (0.3 µmol). After light or dark treatment seedling were harvested under green light and directly transferred in GUS staining buffer. All following steps (vacuum infiltration and 37°C staining) are done in darkness. All seedlings were stained for 8 hours.
A: Schematic illustration of *GUS-SPA1-HA* fusion expressed by SPA1 regulatory sequences or *GUS-SPA2-HA* placed under the control of either *SPA1 or SPA2* regulatory 3´and 5´ regulatory elements.
B: Visual phenotypes of GUS stained wild-type (WT(Col)), *spa1 spa2 spa3 spa4* mutant, *spa1 spa2 spa3* mutant or transgenic seedlings grown in darkness that expressing a fusion of GUS and either SPA1-HAor SPA2-HA under the control of their endogenous promoter *SPA1* and *SPA2. For SPA2 cDNA* a further fusion with GUS is expressed by 5´and3´ regulatory elements of SPA1.
C: Visual phenotype of four additional T2, GUS stained transgenic lines that express *GUS-SPA2* under the control of the *SPA1* regulatory elements.

GUS-*SPA2* expressed by either the *SPA1* or *SPA2* promoter, accumulated to similar levels in the dark, but also in the light (Figure 27 B, C and D). These results show that SPA2 protein produced by the light-inducible *SPA1* promoter accumulates to high levels in the light, but nevertheless cannot replace the function of the SPA1 protein driven by its endogenous regulatory elements. More detailed GUS expression analysis revealed that SPA1-GUS and SPA2-GUS are also expressed more or less in the same tissues (Supplemental Figure 38). Taken together, SPA2 protein is expressed to high levels in the light, but it does not fulfil SPA1-like functions.

III. Discussion

The four-member *SPA* gene family plays important roles in suppression of light signaling. Each of the four SPA genes has distinct, but overlapping functions in different light-influenced developmental switches in the plant life cycle. *SPA1* and *SPA2* control seedling development in the dark, while *SPA1* and, to less extent, *SPA3* and *SPA4* repress photomorphogenesis in light-grown seedlings. In later developmental stages, the functions of *SPA3* and *SPA4* ensure proper vegetative growth and *SPA1* plays an important role in repressing flowering under non-inductive short days. However, the underlying molecular differences among the *SPA* genes that cause their distinct functions are unknown.

III.1. *SPA* gene transcription is under the control of endogenous and exogenous cues

The simplest explanation for the distinct *SPA* gene functions in dark- and light-grown seedlings would be that the transcription of *SPA* genes is directly regulated by the light regime. *SPA1* transcript was previously shown to be more abundant in seedling transferred to FR and R than in the dark (Hoecker et al., 1999). I could show that also the expression levels of *SPA3* and *SPA4* are increased in seedlings that were exposed to FR, R and B. In contrast to that, *SPA2* mRNA levels were unaffected in different light conditions when compared to darkness. Recently, it was also shown that SPA proteins behave very similarly (Fittinghoff et al., 2006; Saijo et al., 2008; Zhu et al., 2008). These results imply, that *SPA1*, *SPA3* and *SPA4* play important functions in light-grown seedlings because their transcripts and proteins are up-regulated in the light. SPA1 protein levels increase fast within one hour, whereas SPA3 and SPA4 protein levels are up-regulated after six hours exposure to light. Like *SPA2* mRNA, its protein is among all SPAs the most abundant protein in darkness. *SPA2* mRNA abundance does not increase in light, but its protein levels exhibit a six fold decrease in light, correlating with SPA2 functions in darkness and not in light. The same situation was found in adult plants: *SPA3* and *SPA4* mRNAs accumulate to high levels in leaves of adult plants, fitting perfectly with their role in regulating adult plant size. However, that cannot explain all distinct *SPA* functions because although *SPA* genes are differentially regulated during the plant life cycle, the total

transcript abundance of the different *SPA* genes does not correlate always. For instance, *SPA4* transcript is high abundant in adult plants, but *SPA1* is almost as strongly expressed as *SPA4* in this developmental stage. These results imply that there must additional higher levels of *SPA* gene regulation that cannot be explained by simple transcript analysis. Zhu et al. showed that SPA2 protein levels are decrease in light-grown seedlings, indicating that there might be post-translational control of SPA proteins as well. Also, SPA proteins accumulate in *cop/det/fus* mutants, implying a possible feed-back-control among negatively acting factors such as SPA and COP proteins (Zhu et al., 2008).

SPA promoter analysis suggests sophisticated spatial promoter activity. In young plants *SPA4* promoters are less active than those of *SPA1* and SPA2 in cotyledons and true leaves*.* A significant difference in spatial *SPA* promoter activity was detectable in the roots. *SPA2* and *SPA1* are strong active in roots, whereas *SPA4* promoter activity was only weak or not detectable. SPA protein data described in Zhu et al., 2008, only partially correlates with *SPA* mRNA results. On protein levels SPA2 is most abundant in roots, but SPA4 and not SPA1 is also strongly expressed in roots suggesting stabilizing posttranscriptional or posttranslational SPA4 modifications or destabilizing mechanism for SPA1 protein. However, I cannot completely exclude the possibility that the chosen *SPA* promoter region do not completely reflect the endogenous *SPA* gene expression. Also differences in growth condition or plant age might effect *SPA* mRNA and protein abundance and make it therefore difficult to compare results from different labs.

SPA promoter analysis in plants after flowering revealed strong promoter activity of *SPA1* and *SPA4* in vascular tissues of older leaves, whereas *SPA2* show a more widespread activity in the whole leaves. Protein data only reflected high SPA4 and SPA1 and less SPA2 protein abundance in leaves (Zhu et al., 2008). Especially this spatial *SPA* regulation might play important roles, as COP1-SPA complex targets like the flowering-time regulator CO are only expressed in specialized cells (An et al., 2004).

SPA transcript and SPA protein levels might also be regulated by environmental cues or tissues not analyzed in this study or in Zhu et al., 2008. Large-scale microarray profiling revealed that *SPA1*, *SPA2* and *SPA4* transcripts are expressed in all 78 tissues and developmental stages that were analyzed (Supplemental Figure 39, *SPA3* is not represented on standard gene expression arrays) (Schmid et al., 2005).

Similar studies revealed, that *SPA* transcripts are not affected by either abiotic or biotic stresses, while all types of light influence *SPA1* and *SPA4* transcript level (Supplemental Figure 40-42) Interestingly, *SPA1* and *SPA2* transcript levels are probably regulated by the plant hormone gibberellic acid (GA), which suggests that some plant hormones might at least partially act through regulation of the *SPA* repressor genes (Supplemental Figure 41).
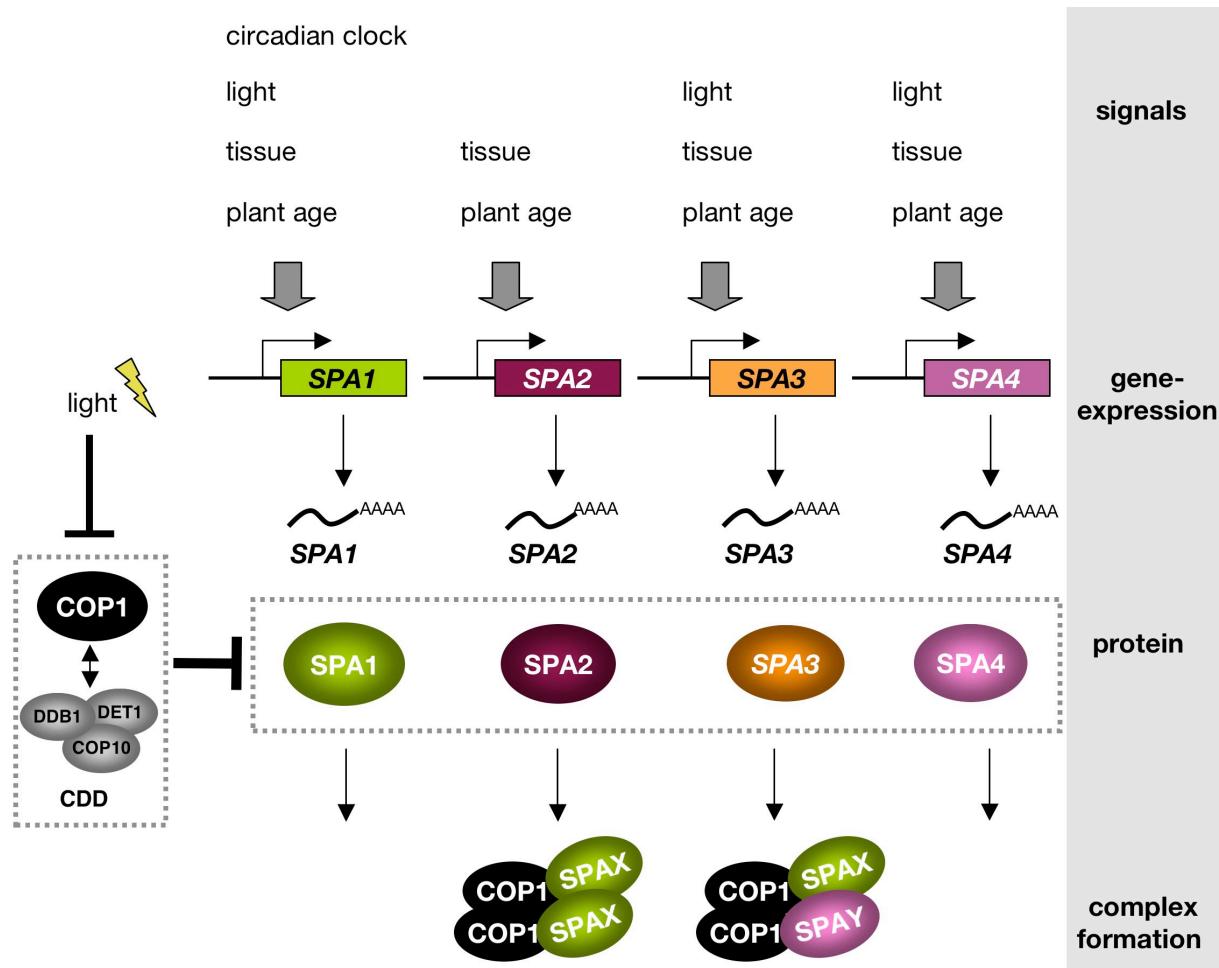
Figure 28: SPA transcript and protein levels correlates partially with its distinct function.

Different endo-and exogenous signals influence *SPA* transcript levels. Also SPA protein levels differ during plant development. Zhu et al., 2008 show that COP1 and parts of the CDD and CSN complex are involved in SPA maintenances. COP1 function is repressed in darkness. Regulation of *SPA* transcript and protein levels leads to certain complex formation, which specifically occurs at different developmental stages.

Taken together, my results and those published by Zhu et al., 2008, imply that there are multiple levels of *SPA* activity regulation (Figure 28). First, *SPA* genes are transcribed differentially depending on light regime, tissue or developmental stage. But also, SPA protein abundance is regulated by the same environmental and

endogenous cues, e.g. light might promote destabilization of SPA2 (Zhu et al., 2008). Last, but not least, differential formation of distinct COP1-SPA complexes in different developmental stages, tissues and growth conditions can be important for regulation of ubiquitination targets.

III.2. *SPA* mRNA abundance is regulated by different photoreceptors

phyA is the only photoreceptor able to sense FR, but phyA is also responsible for R and B signal transduction, especially under low fluence rates. *spa1*, *spa3* and *spa4* single mutants are hypersensitive to FR, R and B light, which mainly relies on functional phyA (Hoecker et al., 1999; Laubinger and Hoecker, 2003). Accordingly, *SPA1*, *SPA3* and *SPA4* transcripts are strongly up-regulated in FR, R and B, but interestingly, this process requires the coordinated action of phyA, the R-sensing phyB and the B-sensing photoreceptors cry1 and cry2. These results imply that all photoreceptors initiate a negative feedback loop that aims to desensitizes light signaling. This is in contrast to COP1: The overall levels of COP1 protein are not affected by light, rather photoreceptors inactivate COP1 either by triggering its nuclear exclusion or by direct physical interaction (Deng et al., 1992; Von Arnim and Deng, 1994; Wang et al., 2001; Yang et al., 2001; Seo et al., 2004; Subramanian et al., 2004). Hence, a contemporaneous activation of the SPA proteins, that are integral components of a functional COP1 complex, might help to enhance the activity of residual COP1 function in the light. In addition, neither the components of the E2-conjugating CDD complex nor the subunits of the COP9 signalosom are affected by light (Pepper et al., 1994; Suzuki et al., 2002; Yanagawa et al., 2004). Hence, photoreceptor regulation of *SPA* transcript abundance might be an important regulatory module for fine-tuning light signaling in young seedlings (Figure 29).

But why is hypersensitivity of *spa* single mutants depended on functional phyA? One possible explanation is that phyA has a different relationship with the COP1/SPA complex than the other photoreceptors. cry1 and phyB are light-stable photoreceptors, while cry2, like phyA, becomes degraded in the light. However, it is not known whether COP1 directly regulates cry2 stability via ubiquitination. In fact, phyA is the only photoreceptor, which was shown to be an ubiquitination substrate of the COP1. Moreover, phyA interacts with SPA1 and becomes rapidly degraded in the light, a process that is delayed in *spa* triple and *cop1* mutants (Seo et al., 2004; Saijo et al., 2008). These results imply that mainly enhanced activity of the photoreceptor

phyA causes hypersensitivity of *spa* mutants in young seedlings. In addition, phyA specific signaling intermediates, like LAF1, FHY1, FHY3 or FAR1, might be targets of SPA containing complexes (Wang and Deng, 2002; Shen et al., 2005; Lin et al., 2007b; Saijo et al., 2008). However, analysis of a *spa1 spa2 spa3 spa4 phyA* quintuple mutant is necessary to completely uncover the role of *PHYA* in *SPA* regulated light signal transduction.
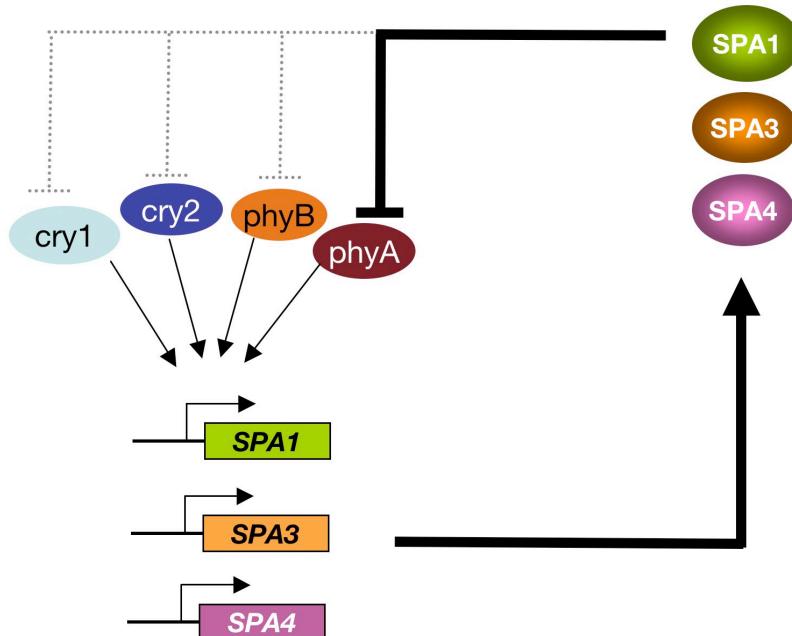
Figure 29: Negative feedback loop between photoreceptors and SPAs are responsible for desensibilization in light signaling.

Light influences *SPA1*, *SPA3* and *SPA4* transcript levels by several photoreceptors. In light SPA1, SPA3 and SPA4 form multiple complexes with COP1 and mediate degradation of phyA.

III.3. All SPA proteins act as repressors in darkness – and some also in the light

In order to investigate whether distinct *SPA* gene functions are due to their different regulatory promoter elements or to their different protein sequences. A promoter-swap experiment with three *SPA* genes, *SPA1*, *SPA2* and *SPA4*. was conducted. These three genes cover all the potential *SPA* gene functions: *SPA1* and *SPA2*, but not *SPA4*, are sufficient for suppression of photomorphogenesis in dark-grown seedlings. *SPA1*, and to weaker extent *SPA4*, inhibit photomorphogenesis in the light. In contrast to that, *SPA2* has almost no function in light-grown seedlings. Furthermore, these three *SPA* genes show also show distinct expression patterns:

The *SPA1* and *SPA4* promoter, but not the *SPA2* promoter, are light responsive. On the other hand, comparative transcript analysis and *SPA* promoter GUS experiments strongly suggest that the promoters of *SPA1* and *SPA2* are stronger than that of *SPA4*.

Expression of the *SPA4* cDNA under the control of the *SPA1* promoter is sufficient to rescue the phenotype of a dark-grown *spa* quadruple mutant. In contrast, *spa1 spa2 spa3* triple mutants that contain only a functional *SPA4* gene driven by the endogenous promoter, undergo photomorphogenesis in complete darkness. This result indicates that the SPA4 protein can also completely suppress photomorphogenesis in the dark when expressed under a stronger promoter, such as the *SPA1* promoter. Hence, all SPA proteins can efficiently act as repressors in the dark as long as they are expressed at certain appropriate levels.

SPA4 also represses photomorphogenesis in light-grown seedlings, although to much lower extent than *SPA1* does. This might be due to the fact that the promoter of *SPA4*, in contrast to that of *SPA1*, is not strong enough to produce sufficient amounts SPA4 to completely suppress photomorphogenesis in the dark. This idea supported by the finding that *SPA4* driven by the *SPA1* promoter is able to repress photomorphogenesis. On the other hand, the *SPA1* cDNA driven by the *SPA4* promoter does not even produce sufficient amounts of SPA1 to avoid de-etiolation in the dark. Again, these findings support the idea that SPA1 and SPA4 can act in the same fashion, in both the dark and the light. Different functions of SPA1 and SPA4 in wild-type plants are mainly due different promoter activities and strengths.

This model cannot to be transferred to *SPA2*. Endogenous *SPA2* is functional in the dark, but not in the light. Placing *SPA2* under the control of the light-inducible *SPA1* promoter does not convert SPA2 protein into a repressor that can also function in light-grown seedlings. However, seedlings expressing SPA2 under the control of the *SPA1* promoter undergo normal skotomorphogenesis in the dark, indicating that SPA2 is fully active. In addition, protein GUS fusion experiments rule out that the SPA2 protein is degraded in the light, because the GUS-SPA2 protein accumulates in dark- and light-grown seedlings.

Taken together, these results suggest that all SPA proteins can act as repressors in the dark. In the light, however, the SPA2 protein is not functional. But why do SPA1 and SPA4 function in the light, but SPA2 does not? *SPA1* and *SPA2* exhibit almost equal size and show conserved locations of all splice sites. Both genes arose from a

duplication event in the Arabidopsis genome; and their proteins exhibit a long N-terminal region. In contrast to that, SPA4 and SPA1 only share high sequence similarity within the WD-repeats and SPA4 features only a very short N-terminal domain.

One possible explanation is that SPA proteins require a post-translational modification for activity in the light. The SPA2 protein might lack a crucial amino acid necessary for such an activating modification (Figure 30 A). Alternatively, SPA2 might undergo post-translational processing events in the light that efficiently block its activity. In such a scenario, one would claim that SPA1, SPA3 and SPA4 lack crucial amino acids for such a repressive modification (Figure 30 B). No matter what the SPA2 protein features, it might interfere with COP1/SPA complex formation in the light. In the dark, SPA2 is associated with COP1 and the other SPA proteins. These interactions are almost completely abolished in light-grown seedlings. Hence, post-translational processing events might target SPA2 ability to interact with COP1 and other SPA proteins. Alternatively, SPA2 containing COP1 complexes are more repressible by light. In such a scenario, COP1-SPA2 complexes dissociate more easily than other COP1-SPA complexes (Figure 30 C). In the future, random mutagenesis approaches, as well as domain-swaps or domain-deletions-derivates of SPA2 will help to identify crucial amino acids that can explain the different functions of SPA proteins in the dark and the light.

Possible candidates for mediating post-translational modification might be the photoreceptors themselves. Photo-activated phyA induces phosphorylation of its interacting partners PIF1, PIF3 and PIF5 (Al-Sady et al., 2006; Castillon et al., 2007). NPH3, a component of an E3 ubiquitin ligase complex, undergoes goes blue-light dependent dephosphorylation mediated by phot1 (Motchoulski and Liscum, 1999; Pedmale and Liscum, 2007). Apart from post-translational, light-induced conformational changes in the crys are responsible for blocking COP1 activity in response to B. Because SPA1 interacts with phyA and possibly also via its WD-repeat domain with crys, future research might focus on SPA protein modifications mediated by different photoreceptors.

A. Light dependent modifications activate SPA function:
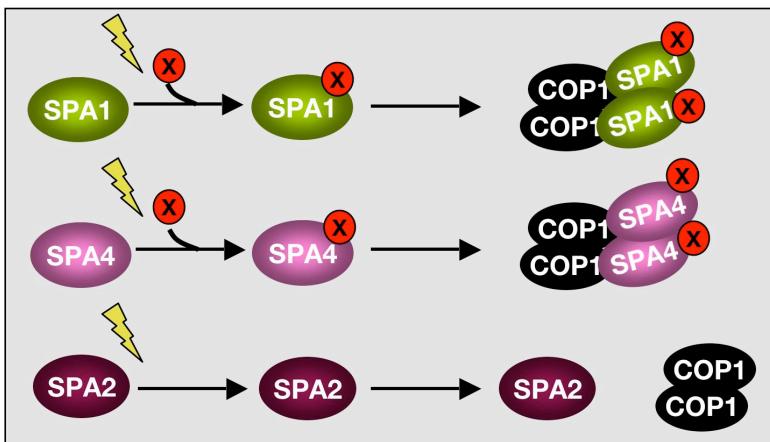
B. Light dependent modifications repress SPA2 activity:
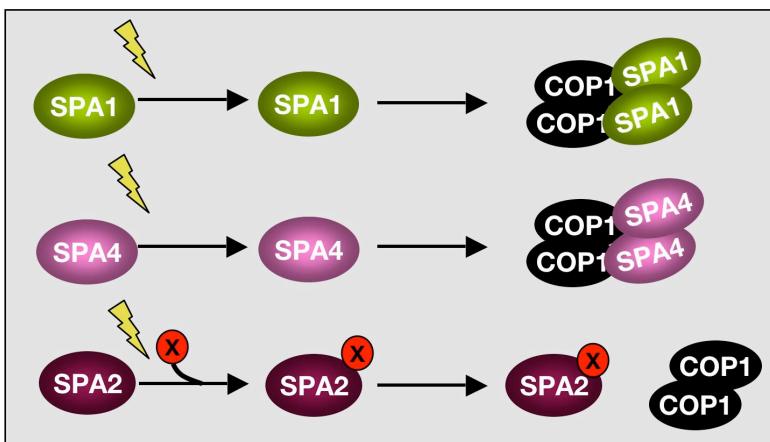
C. Unequal repression of different COP1-SPA complexes by light:
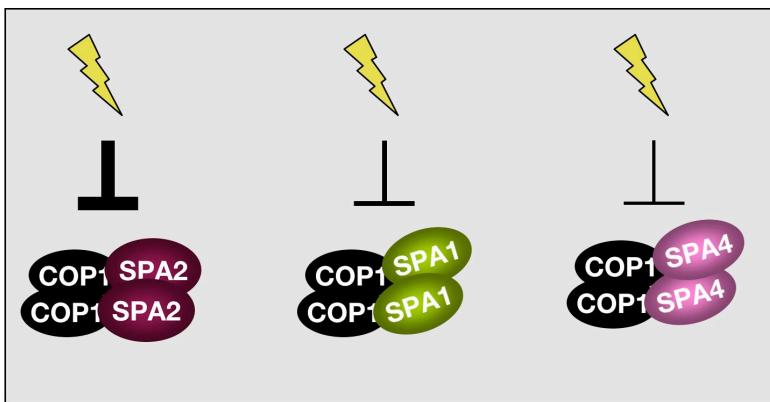

Figure 30: Possible light-depended SPA protein modifications might influence SPA function.

A: SPA1 and SPA4 but not SPA2 might be activated by protein modifications in light.
B: SPA2 but not SPA1 and SPA4 might be repressed by protein modifications in light
C: SPA2-containing complexes are easier repressible by light.

III.4. The SPA N-terminus has an important function in suppression of flowering

SPA proteins act in concert with the ubiquitin ligase COP1. Recent findings suggest that a COP1 dimer forms heterogeneous complexes with two other SPA proteins (Zhu et al., 2008). However, it is still an open question what function the SPA proteins play in the COP1-SPA complex. SPA1 can affect COP1 ubiquitin ligase activity in vitro, and SPA proteins might also affect COP1 sub-cellular localization (Saijo et al., 2003; Seo et al., 2003). Meanwhile, it is well established that SPA proteins can directly interact with diverse ubiquitination substrates. SPA1 binds to LAF1, HFR1, HY5, CO and phyA in vivo and the protein levels of all these proteins are increased in *spa* mutants (Saijo et al., 2003; Seo et al., 2003; Seo et al., 2004; Yang et al., 2005a; Laubinger et al., 2006). These results indicate that SPA proteins are responsible for substrate recognition. For efficient binding of HY5 and HFR1, SPA1 requires its WD-repeat domain and its coiled-coil domain. The coiled coil domain also mediates interactions with other SPA proteins and COP1 (Hoecker and Quail, 2001; Saijo et al., 2003). However, the function of the N-terminal domain of SPA1, that includes a kinase-like motif, is unknown. Therefore, I asked whether the N-terminal domain is generally necessary for SPA1 function. Expression of a SPA1 protein lacking the kinase-like domain or even the whole N-terminus can completely rescue the phenotype of *spa1* single or *spa1 spa2 spa3* triple mutants. However, COP1-SPA complexes can contain SPA heterodimers, suggesting that truncated SPA1 proteins expressed in a *spa1 spa2 spa3* triple mutant can form heterodimers with SPA4 (Figure 31). In addition, Zhu et al., 2008, recently showed that the *spa2-1* mutant allele produces detectable levels of a truncated SPA2 protein that still features its N-terminal domain. Therefore, I cannot completely exclude that SPA1 also forms heterodimers with the truncated SPA2 protein and that the resulting COP1-SPA complex still harbors a functional SPA N-terminus. The same approach should be repeated in a *spa* quadruple mutant, generated with true *spa* null alleles.

However, the SPA1 protein lacking the kinase-like domain or the whole N-terminus cannot rescue the early flowering phenotype of *spa1* in short days. These results indicate that the SPA1 N-terminus has an essential role for proper SPA1 function (Figure 31). SPA1 protein lacking the N-terminus accumulates to even higher levels than the full-length protein, while their respective transcripts remain unchanged (Yang and Wang, 2006). This observation rules out the possibility that truncated

SPA1 proteins do not accumulate to endogenous full length SPA1 levels and also strongly reinforces the idea, that SPA proteins are also regulated by post-translational mechanisms. But why can the SPA1 protein without N-terminus complement the *spa1* phenotype in seedlings and not in adult plants?

SPA1 and COP1 repress flowering in short days by decreasing CO protein stability (Jang et al., 2008). *spa2 spa3 spa4* triple mutants flower like wild type, indicating that SPA1 alone is sufficient for suppression of CO function (Laubinger et al., 2006). Therefore, SPA1 might have a very high affinity to CO in vivo and, therefore, SPA1 N-terminus might contribute to substrate recognition.

It is also tempting to speculate that SPA proteins have special tissue-specific functional requirements. CO promotes flowering only in specific cells, the phloem companion cell of the vascular bundles. Probably, an unknown co-factor specifically binds or modifies the SPA1 N-terminus that is expressed only in these cell types. It is already known that specific factors act in the vascular bundles, such as CRY2 to regulate flowering or PRR3 (Para et al., 2007) to regulate the circadian clock.
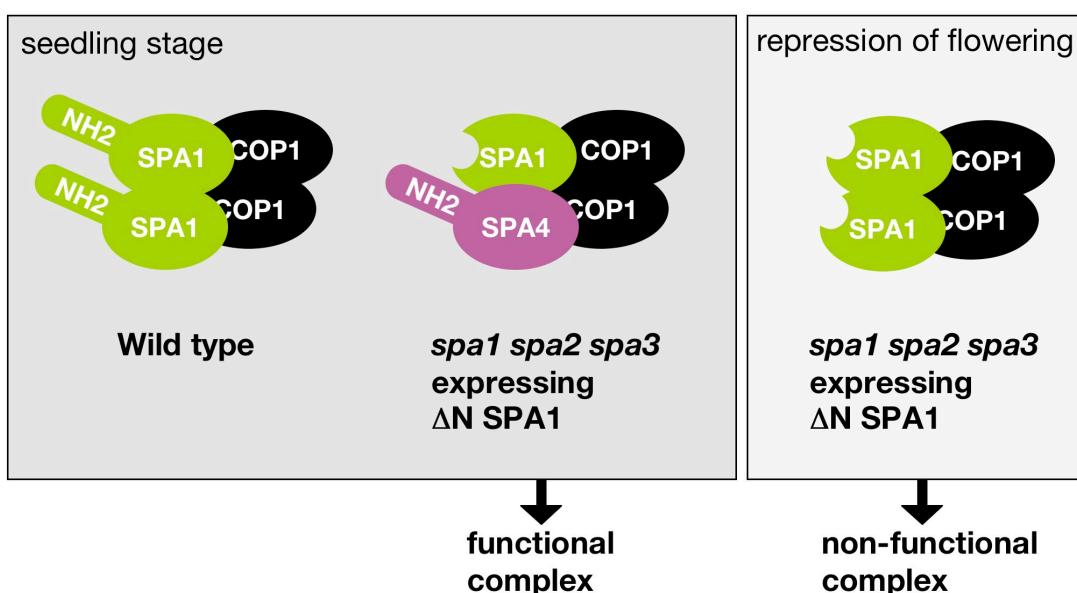
Figure 31: Possible models for the functions of SPA N-terminus in COP1-SPA complexes.

COP1-SPA complexes featuring two or at least one SPA N-terminus are functional. In contrast to that, COP1-SPA complexes without any SPA N-terminus do not have regulatory activity.

Alternatively, SPA1 might be much more abundant than the other SPA proteins in these cells. Thus, in these cells I would expect a predominant formation of COP1-SPA1 complexes containing a COP1 homodimer and a SPA1 homodimer. In such a

scenario, I would not expect the SPA1 protein without N-terminus rescue the *spa1* mutant phenotype, when at least a single SPA1 N-terminus is essential for COP1-SPA complex function.

An important unanswered question is whether SPA proteins exhibit kinase activity. If so, one could also think that some substrates, like CO, are phosphorylated prior to degradation. The transcription factor HFR1 was shown to be phosphorylated and that the phosphorylated HFR1 becomes degraded very rapidly. However, it is not known whether SPA proteins are responsible for HFR1 phosphorylation. In addition, phosphorylation of HY5 has contrary effects and stabilizes the HY5 protein. Nonetheless, the fact that SPA1 N-terminus is indispensable for SPA1-mediated regulation of flowering time raises the question about the biochemical function and the biological importance of the SPA N-terminus.

IV. Materials and Methods

IV.1. Materials

IV.1.1. Chemicals and antibiotics

Chemicals and antibiotics were obtained from the companies with research grade "p.a." or the highest available purity:

Ambion (Austin, USA), Applichem (Darmstadt, Germany), Colgate-Palmolive (Hamburg, Germany), Duchefa (Haarlem, Netherlands) Difco (Detroit, USA), Fluka AG (Buchs, Switzerland), Gibco BRL (Eggenstein, Germany), Invitrogen (Karlsruhe, Germany), MBI Fermentas (St. Leon-Rot, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) Riedel-de-Haen (Seelze), Serva (Heidelberg, Germany) and Sigma-Aldrich (Deisenhofen, Germany).

IV.1.2. Radioactivity, enzymes kits and antibodies

α-[^{32}P]-dATP was delivered from Amersham Pharmacia Biotech (Uppsala, Sweden). Enzymes for molecular biology were obtained from following companies:

MBI Fermentas (St. Leon-Rot, Germany), Clontech (Palo Alto, USA), Invitrogen (Karlsruhe, Germany), Roche (Mannheim, Germany) and New England Biolabs (Ipswich, USA).

The following kits were used according to the manufactures' protocols: Prep Plasmid Midi (Qiagen GmbH, Hilden, Germany) QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany).

In this study, α HA antibody from Roche (Mannheim, Germany) und α Tubulin from Sigma-Adlrich (Deisenhofen, Germany) were used for immunodetection.

IV.1.3. Bacterial strains

For standard cloning, *Escherichia coli* strain *DH5α* was used. For gateway cloning of destination vectors, the *ccdB* gene resistant *Escherichia coli* strain *DB3.1* (Invitrogen) was used. *Agrobacterium tumefaciens* strains *GV3101 (pMP90RK)* was used for all plant transformations.

IV.1.4. Oligonucleotides

All oligonucleotides were synthesized by Invitrogen Life Technologies (Karlsruhe, Germany) or Metabion (Martinsried, Germany).

Table 1: Sequences of all oligonucleotides that were used in this thesis and their purpose

Oligo-nucleotides	Sequences (5´to 3`)	Application
ApaI-cS1-F1	TGA**GGGCCC**ATGCCTGTTATGGAAAGAGTA ApaI	cloning of promoter swap constructs pSPA1::cSPA1-HA; pSPA2::cSPA1-HA; pSPA4::cSPA1-HA
ApaI-cS2-F2	TGA**GGGCCC**ATGATGGATGAGGGATCAGTA ApaI	cloning of promoter swap constructs pSPA1::cSPA2-HA; pSPA2::cSPA2-HA
ApaI-cS4-F4	TGA**GGGCCC**ATGAAGGGTTCTTCAGAATCT ApaI	cloning of promoter swap constructs pSPA4::cSPA4-HA; pSPA1::cSPA4-HA
cS1-NotI-R1	AGCT**GCGGCCGC**AACAAGTTTTAGCTT NotI	cloning of promoter swap constructs pSPA1::cSPA1-HA; pSPA2::cSPA1-HA; pSPA4::cSPA1-HA
cS2-NotI-R2	AGCT**GCGGCCGC**CTGGTTGACATCTTGAAAACT NotI	cloning of promoter swap constructs pSPA1::cSPA2-HA; pSPA2::cSPA2-HA
cS4-NotI-R4	AGCT**GCGGCCGC**TACCATCTCCAAAATCTTGAT NotI	cloning of promoter swap constructs pSPA4::cSPA4-HA; pSPA1::cSPA4-HA
KpnI-S1Pro-F	GTCA**GGTACC**CATGTTGCTGGTTAGGTTGA KpnI	cloning of promoter swap constructs pSPA1::cSPA1-HA; pSPA1::cSPA2-HA; pSPA1::cSPA4-HA
KpnI-S2-Pro-F2	GTCA**GGTACC**ATACTGCAAACGCAAATTGG KpnI	cloning of promoter swap constructs pSPA2::cSPA2-HA; pSPA2::cSPA1-HA
KpnI-S4-Pro-F4	GTCA**GGTACC**ATGATCTTCTTGGACATGCA KpnI	cloning of promoter swap constructs pSPA4::cSPA4-HA; pSPA4::cSPA1-HA
S1Pro-ApaI-R	GACT**GGGCCC**CAACACTCATTGCATCAGCA ApaI	cloning of promoter swap constructs pSPA1::cSPA1-HA; pSPA1::cSPA2-HA; pSPA1::cSPA4-HA
S2-pro-ApaI-R2	GACT**GGGCCC**TCTCTGTATCATAGGAAACATA ApaI	cloning of promoter swap constructs pSPA2::cSPA2-HA; pSPA2::cSPA1-HA

Oligo-nucleotides	Sequences (5´to 3`)	Application
S4-pro-ApaI-R4	ACT**GGGCCC**TGATTACCAAACAAAACTAAAACTCCT ApaI	cloning of promoter swap constructs *pSPA4::cSPA4-HA; pSPA4::cSPA1-HA*
3`UTR SPA1 kpnIsacI-R1	CT**GAGCTCGGTACC**CAATGTACCCATTACATGTTGGA SacI KpnI	cloning of promoter swap constructs *pSPA1::cSPA1-HA; pSPA1::cSPA2-HA; pSPA1::cSPA4-HA*
3`UTR SPA2 kpnIsacI-R2	CT**GAGCTCGGTACC**ACCTTACTATGTATACCCATCA SacI KpnI	cloning of promoter swap constructs *pSPA2::cSPA2-HA; pSPA2::cSPA1-HA*
3`UTR SPA4 kpnIsacI-R4	CT**GAGCTCGGTACC**TCAAGCAGCTTGGAGAGAATCT SacI KpnI	cloning of promoter swap constructs *pSPA4::cSPA4-HA; pSPA4::cSPA1-HA*
NotI-3`UTR SPA1-F1	CCTA**GCGGCCGC**CAACTTGGCCTTTTCGCTACTACT NotI	cloning of: *pSPA1::cSPA1-HA; pSPA1::cSPA2-HA; pSPA1::cSPA4-HA*
NotI-3`UTR SPA2-F2	CCTA**GCGGCCGC**AATAAATACATACATACAGAAGAAGC NotI	cloning of: *pSPA2::cSPA2-HA; pSPA2::cSPA1-HA*
NotI-3`UTR SPA4-F4	CCTA**GCGGCCGC**ACCAATATAAAGTGGCTCAG NotI	cloning of: *pSPA4::cSPA4-HA; pSPA4::cSPA1-HA*
ApaI-GUS-F11	CTGA**GGGCCC**ATGTTACGTCCTGTAGAAACCCCAACC ApaI	Cloning GUS into ApaI site of promoter–swap constructs
GUS-APAI-R11	GA**GGGCCC**TTGTTTGCCTCCCTGCTG ApaI	Cloning GUS into ApaI site of promoter–swap constructs
3xHA-NotI-F	CCTA**GCGGCCGC**TTACCCATATGACGTTCCAGAC NotI	Cloning of promoter-swap and *SPA1* deletion constructs
3xHA-NotI-TGA-R	GGTA**GCGGCCGC**TCAAGCGTAGTCAGGTACGTCGTAAG NotI Stop	Cloning of promoter-swap and *SPA1* deletion constructs
MfeI-SphI-F	AATTGAAAGCACGAGTGGAGATGCATG	Cloning of *SPA1* deletion construct *ΔN*
MfeI-SphI-R	CATCTCCACTCGTGCTTTC	Cloning of *SPA1* deletion construct *ΔN*
SPA1-F	ACTGCAAACAGTGATTGTC	Cloning of *SPA1* deletion construct progenitor *and FL SPA1*
Δ KIN-R	CCATGTGGACATAGACTG	Cloning of *SPA1* deletion construct *ΔKIN*
Δ KIN-F	CAGTCTATGTCCACATGGGATGATTCAGTTAAATCG	Cloning of *SPA1* deletion construct *ΔKIN*
SPA1-R	ACACCATCGTAGTCAGTCGACG	Cloning of *SPA1* deletion construct progenitor and *FL SPA1*
SPA1-F1-UH	TGATTTAAACATGGTTGATGCACG	Cloning of *SPA1* deletion construct progenitor and *FL SPA1*
Δ cc-R	TTTAGATGCTTTTTTCTTCTTC	Cloning of *SPA1* deletion construct *Δcc*
Δ cc-F	GAAGAAGAAAAAAGCATCTAAAATGCGATCACAAATCAACTTA	Cloning of *SPA1* deletion *Δcc*

Oligo-nucleotides	Sequences (5´to 3`)	Application
HA-R	CTTACGACGTACCTGACTAC	RT-PCR
SPA1-F-KF	TTGTCATGAGAAAGCGGTGA	RT-PCR
pro-S4+1040	GATCGTTTCTTCGGTGCCTCTTAGTC	Co-segregation analysis *pSPA4::cSPA1-HA*
SPA1-R16	ACAGAAAGCTTAGCTCCTGCGGCTTC	Co-segregation analysis *pSPA4::cSPA1-HA*
msa6-F	CTGGGGTGTTCTCACAGGAT	Co-segregation analysis control
msa6-R	TGAATTCGGTTCAAGATTGT	Co-segregation analysis control
UBQ10-F	CTGTTATGCTTAAGAAGTTCAATGT	RT-PCR; UBQ10 probe for RNA blot
UBQ10-R	GAAACATAGTAGAACACTTATTCATC	RT-PCR ;UBQ10 probe for RNA blot
pSPA1 F (Aashish Ranjan)	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAAATAATACAACATGTTGCTGGT	Promoter SPA1::GUS
pSPA1 R (Aashish Ranjan)	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAACAGGCATCAACACTCATT	Promoter SPA1::GUS
pSPA2 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTTGCATTTGAATACTGCAAA	Promoter SPA2::GUS
pSPA2 R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCCATCCATCATTCTCTCTG	Promoter SPA2::GUS
pSPA4 F (Aashish Ranjan)	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAATGATCTTCTTGGACATGCATC	Promoter SPA4::GUS
pSPA4 R (Aashish Ranjan)	GGGGACCACTTTGTACAAGAAAGCTGGGTTTGATTACCAAACAAACTCCTCT	Promoter SPA4::GUS
LB-3	TAGCATCTGAATTTCATAACCA	genotypisierung *spa4-1*/WT
SPA4-R11	TGAAGCAATAGAAACGAATCTCG	genotypisierung *spa4-1*/WT
SPA4-F11	TTAACGGTTGAGTTCGTTTTCC	genotyping *spa4-1*/WT
4-R7	CCAAATGCTAAAGACCGACCCGTC	distinguish cSPA4 from gSPA4
4-F7	GTAACTTTGAAGGCGTGGTTCAAG	distinguish cSPA4 from gSPA4

Oligonucleotides used for verification of *SPA* constructs via sequencing or colony PCR are listed in the Vector-NTI-database of the Hoecker lab.

IV.1.5. Cloning vectors

pBluescript KS (pBs; Stratagene, La Jolla, USA) was used for standard clonings. Blunt-end cloning vector pJET (MBI-Fermentas, St. Leon-Rot, Germany) was used for facilitate blunt-end sub-cloning of PCR fragments. Entry vector pDONR221 was used for BP reactions for the *SPA* promoter analysis.

Binary vector pzp212 (spectinomycin resistance gene, kanamycin resistance gene) and its modified successor pJHA 212 were used for Agrobacterium transformation. A modified pJHA 212 (kanamycin resistance gene *npt* was replaced by the hygromycin resistance gene *hpt*) was kindly provided by Ute Höcker. Binary destination vector pGWB3 that includes the GUS reporter gene was used for *SPA* promoter analysis (Nakagawe et al.,2007).

IV.1.6. Plant lines

The *spa1-3* mutant allele was derived from an EMS mutagenesis (RLD background) and carries a single base pair substitution that leads to a premature stop codon in the first exon (Hoecker et al., 1999). The *spa1-100* mutant was generated in the Col background and likely represents a *spa1* null allele (Yang et al., 2005). This mutant carries a T-DNA insertion in the second Exon of *SPA1* gene and was isolated from The Syngenta Arabidopsis Insertion Library (SAIL) T-DNA insertion mutant population (Session et al., 2002). *spa3-1* carries a T-DNA insertion in its first Intron, whereas two T-DNAs are inserted head-to head 3´to the codon for D640 of the *SPA4* protein in *spa4-1*. *spa3 spa4, spa1 spa2 spa3*, and *spa1 spa2 spa3 spa4* multiple mutants are described elsewhere (Laubinger and Hoecker, 2003; Fittinghoff et al., 2006). The *phyA-101* allele is a *phyA* null allele in the RLD ecotype (Dehesh et al., 1993). The *phyB*, *phyA phyB*, *cry1 cry2*, *phyA cry1 cry2* mutants were described previously (Hoecker et al., 1998; Mazzella et al., 2001).

IV.2. Methods

IV.2.1. Seed sterilization

For sterile growth of Arabidopsis on MS-plates, seeds were surface sterilized. For dry seed sterilization, aliquots of seeds were incubated with chlorine gas. To produce chlorine gas, 80 ml of sodium hypochloride was mixed with 2.5 ml of concentrated

hydrochloric acid in an exsiccator. Aliquots of seeds were incubated for approximately 4 hours.

Liquid sterilization was applied when seedling were used for hypocotyl length measurements. Therefore, seeds were surface-sterilized with 20% Klorix (Colgate-Palmolive, Hamburg, Germany) and 0.03% Tween-20 for ten minutes, washed five times with sterile water, and plated on 1xMS medium without sucrose.

IV.2.2. Plant growth

Arabidopsis seeds were stratified in 4°C for three days in water supplemented with 0.1 % agarose. Seeds were normally sown in a substrate mixture containing three parts soil and one part Vermiculit. In the greenhouse, plants were grown under long day conditions with 16 hours light and 8 hours darkness and a relative humidity of approximately 40%. The temperature was kept at 21°C during light period and was reduced to 18°C during darkness. For seedling analysis seeds were sown on sterile MS plates and stratified at 4°C for 4 days, followed by a 3-h white-light treatment at 21°C to induce germination. Plates were kept for 21 h in the dark at 21°C and were then exposed to Rc, FRc, Bc, or darkness for 3 days. Light conditions were generated using LED light sources (Quantum Devices, Barneveld, WI, USA).

To determine the flowering time, seeds were sown directly onto soil and plants were grown in a randomized fashion in SD (8 hours light/16 hours darkness) at 21°C. The light sources were fluorescent tubes (80 μmol m^{-2} s^{-1}).

IV.2.3. Measurement of hypocotyl length

To determine hypocotyl length seedlings were pressed lengthwise in MS media containing 1% agar and documented with a digital camera. Measurements of hypocotyl length were conducted on digital images via NIH Image Software (Bethesda, USA). Statistical analyses were performed via KaleidaGraph 3.6 (Synergy Software) software program.

IV.2.4. Measurement of flowering time

Time of flowering under short days was determined by counting the numbers of true leaves at that day first inflorescence was visible by eye. 10 to 15 plants were analyzed for each genotype. Statistical analyses were made with software program KaleidaGraph 3.6 (Synergy Software).

IV.2.5. Plasmid DNA preparation of bacteria

Plasmid DNA from E.coli in Miniprep scale was isolated using the QIA-prep Spin Miniprep Kit (Qiagen Hilden, Germany) following the manufacturer's protocols.

IV.2.6. Genomic DNA preparation

For Co-segregation analysis, genomic DNA was isolated from single seedlings. Whole seedling was ground to a fine powder under liquid nitrogen and resuspended in 20 µl extraction buffer (50 mM Tris/HCL pH 7.2 and 10% sucrose). For PCR amplification, 1 µl was used as template in a 25 µL PCR reaction.

For high-throughput DNA analysis (e.g. verifying of the transgene in transgenic plants) approximately 15 seedlings of each line were transferred into 8 tube strips (Tissue-lyser compatible, Qiagen Hilden, Germany). Then, 300 µl extraction buffer and a 5 mm stainless steel bead were added. The strips were inserted into the adapters of the tissue lyser and shaked at 30 Hz for 90 sec. 1 µL of the solution was used as template for a 25 µL PCR reaction.

Genomic DNA for amplification cis-regulatory elements for cloning was harvested from reproductive tissue (buds) and purified with DNeasy kit according to manufacturer's protocol (Qiagen Hilden, Germany).

IV.2.7. RNA isolation and Northern analysis

To analyze light regulation of *SPA* transcript levels, total RNA was isolated using the RNeasy plant mini kit (Qiagen, Hilden, Germany) from 4-day-old dark-grown seedlings that had been transferred to the indicated light conditions for 0-30 hours. Five to 15 µg total RNA was separated by standard glyoxal gel electrophoresis and blotted onto nylon membranes. Membranes were hybridized with *SPA1-*, *SPA2-*, *SPA3-* or *SPA4*-specific, ^{32}P-labelled probes comprising the complete respective ORF. Prior dot blot hybridizations had confirmed that the probes are gene-specific. After over night hybridization, the membranes were washed at 65°C once with 2x SSC, 0.1% SDS, once with 0.5x SSC, 0.1% SDS, and once with 0.1x SSC, 0.1% SDS. Exposition to phosphoimager plates was carried out for at least 4 days. Signals of *SPA1*, *SPA2*, *SPA3* or *SPA4*, respectively, were normalized to the signal of *18S rRNA*.

For comparative *SPA* transcript analysis (Figure 5), I used polyA$^+$ RNA rather than total RNA because the separation behaviour during electrophoresis varied among the four *SPA* transcripts when rRNAs were present. First, total RNA was isolated from at

least 5 g of tissue by standard phenol/chloroform extraction followed by lithium chloride precipitation. This total RNA was subsequently used for polyA$^+$ isolation with the oligotex mRNA midi kit (Qiagen, Hilden, Germany). One µg of polyA$^+$ RNA was separated, blotted, and hybridized as described above. For normalization, an *UBIQUITIN10* (*UBQ10*) -specific probe was synthesized by PCR using the primers UBQ10-F and UBQ-R. This probe was used to rehybridize the membranes. *SPA* signals were normalized with the respective *UBQ10* signals and the obtained ratio was further divided by a factor that corrected for differences in probe size (*SPA1*: 3.09 kb, *SPA2*: 3.11 kb, *SPA3*: 2.54 kb, *SPA4*: 2.39 kb). All experiments were repeated at least twice.

IV.2.8. Semi-quantitative RT-PCR analysis

Total RNA was isolated from the green parts of soil-grown plants (three weeks old) at different Zeitgebers (ZT 2, 8, 14, 20) using the RNA Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One µg of RNA was treated with RNase-free DNase I (MBI Fermentas, St Leon-Rot, Germany), according to the manufacturer's instruction and subsequently reverse transcribed using an oligo-dT primer and RevertAid H Minus M-MuLV Reverse Transcriptase (MBI Fermentas, St Leon-Rot, Germany). For PCR 2 µl cDNA was used as template. *SPA1-HA* and *UBQ10* fragments were amplified using gene-specific primers. The *UBQ10* fragment was used as a control to normalize the amount of cDNA used. A *SPA1-HA* fragment was amplified using SPA1-F and HA-R. For all cDNAs, the exponential range of amplification was determined experimentally. Semi-quantitative RT-PCR products were analyzed on agarose gels after 28 (*SPA1-HA*) and 23 (*UBQ10*) PCR cycles.

IV.2.9. Protein isolation and immunoblot analysis

Seedlings were ground in liquid nitrogen, resuspended in protein extraction buffer (150 mM NaCl; 50 mM Tris, pH 7.5; 1 mM EDTA; 10 mM NaF; 25 mM ß-glycerophosphate; 2 mM sodium orthovanadate; 0.1 % (v/v) Tween-20; 10 % (v/v) glycerol, 1 mM DTT; 1 mM PMSF; 2x Complete Protease Inhibitor Cocktail, Roche) and clarified by centrifugation. After determination of the protein concentration using Bradford reagent (Biorad Protein Assay, Biorad), 20-30 µg of total protein was separated by SDS-PAGE and blotted onto nitrocellose membranes. HA-tagged proteins were detected with anti-HA monoclonal antibodies (Roche: Mannheim; Germany). A tubulin-specific antibody (Sigma Aldrich: Deisenhofen, Germany) was

used as a loading control. Chemiluminescence visualization was carried out with the ECL plus Western Blot Detection kit (Amersham).

IV.2.10.   Histochemistry

GUS activity was determined as described previously with some minor modifications (Jefferson et al., 1987). For complete infiltration of the substrate (X-Gluc), plant tissues were vacuum infiltrated in staining buffer (0.1% TritonX-100, 10 mM EDTA (pH 7) 0.5 mM $NaPO_4$ (pH 7.0), 0.5 mM potassium-ferrocyanide ($K_4Fe(CN)_6$+ H_2O), 0.5 mM potassium ferricyanide ($K_3Fe(CN)_6$)) containing 1 mM 5-bromo-4-chloro-3-indolyl-_-d-glucuronic acid (X-Gluc; Duchefa) two times for approximately 15 minutes. Tissues were incubated for two to 16 hours at 37°C. Reaction was stopped by adding 70% ethanol. Chlorophyll was removed by several washing steps with 70% ethanol.

IV.2.11.   DNA manipulation

DNA cloning manipulation and cloning was performed by using standard protocols (Sambrook and Russell, 2001). Correctness of PCR generated cloned fragments was determined by sequencing (AGOWA, Berlin; GATC (Konstanz) and University of Cologne (Department of Genetics). Constructs were designed by using Vector NTI-suite software (Invitrogen).

IV.2.12.   Gateway cloning

BP reaction and LR reaction were performed according to manufacture's protocol (Invitrogen).

IV.2.13.   Plant transformation

Agrobacteria transformation was performed as described previously (Clough and Bent, 1998).

IV.3. Cloning strategies

IV.3.1. Promoter-swap constructs

For the generation of promoter-swap constructs, the same cloning strategy was applied for all constructs. All performed amplifications for cloning were done with recombinant Pfu polymerase (MBI-Fermentas; St Leon-Rot, Germany). Cloning steps were controlled by blue-white selection, colony PCR, and restriction analysis.

First 5´genomic regulatory sequences of *SPA1*, *SPA2* and *SPA4* were amplified from genomic DNA (Col) with primers introducing a 5´ KpnI and a 3´ApaI restriction site (SPA1: -2260 bp; SPA2: -1958 bp and SPA4: -1309 bp before ATG; Table 1). Those PCR products were digested with KpnI and ApaI and ligated into pBluescript (pBS KS; Stratagene). pBS plasmids carrying 5´regulatory sequences of *SPA1*, *SPA2* or *SPA4* (*SPA1*, *SPA2* or *SPA4* promoters) were sequenced.

Second, 3´regulatory sequences of *SPA1*, *SPA2* and *SPA4* were amplified from genomic DNA (Col) with forward primers carrying NotI recognition site and reverse primers carrying a KpnI site followed by SacI restriction site (3´regulatory sequence of *SPA1*+ 672 bp after Stop, *SPA2* +812 bp after Stop, *SPA4* +296 bp after stop codon; Table 1). After PCR amplification, resulting amplicons were digested with NotI and SacI and ligated into pBS carrying the 5' region of *SPA1*, *SPA2* and *SPA4*, respectively. Resulting pBS vectors carrying *SPAx* 5' and *SPAx* 3´regulatory sequences were used for further cloning.

Complementary DNAs (cDNAs) of *SPA1*, *SPA2* and *SPA4* were amplified from previous described constructs: *cSPA1* derived from amplification with FL SPA1 used as a template (Fittinghoff et al. 2006). *cSPA2* was amplified from 35S::GUS-cSPA2 (Laubinger et al., 2004). *SPA4* cDNA was derived from PCR amplification with cSPA4-TOPO as a template (kindly provided by Ute Hoecker). *SPA* cDNAs were amplified without their stop codons with forward primers introducing a 3´ ApaI recognition site and a 5´ NotI restriction site. After PCR amplification resulting amplicons were directly ligated into pJET vector (MBI-Fermentas) and sequenced. After sequencing, *SPA* cDNAs without stop codons were digested with ApaI and NotI and ligated into the ApaI and NotI site of pBs contain the *SPA* 5' and 3' regulatory sequences resulting in following constructs *pSPA1::cSPA1, pSPA2::cSPA2, pSPA4::cSPA4, pSPA1::cSPA2, pSPA1::cSPA4, pSPA2:cSPA1 and pSPA4::cSPA1*.

Those constructs were digested with NotI. With HA specific primers carrying both NotI recognition sites a triplicate of HA was amplified with an artificial stop codon at the end of its sequence. Amplification product was digested with NotI and ligated into all mentioned Promoter-Swap plasmids. HA insertion was verified by restriction analysis and sequencing.

All Promoter-Swap constructs in pBS were digested with KpnI and ligated into pJHA212-hpt (Figure 32). Plasmid clones carry the promoter swap constructs in same orientations as the Mas promoter were selected for Agrobacterium GV3101-

mediated transformation of *spa1-100*, spa3 spa4, or *spa1 spa2 spa3 spa4*/*SPA4* mutants plants by floral dip (Clough and Bent, 1998) Transgenic seeds were selected by plating on 1 x MS medium containing 1% sucrose and 20 mg /L hygromycin (Invitrogen). The presence of the transgene was further confirmed by PCR using primers combination specific to the analyzed transgene. Presence of *pSPA1::cSPA1-HA, pSPA2::cSPA2-HA* and *pSPA4::cSPA4-HA* transgenes was verified by PCR amplification with HA-specific primer and *SPA* specific primers.
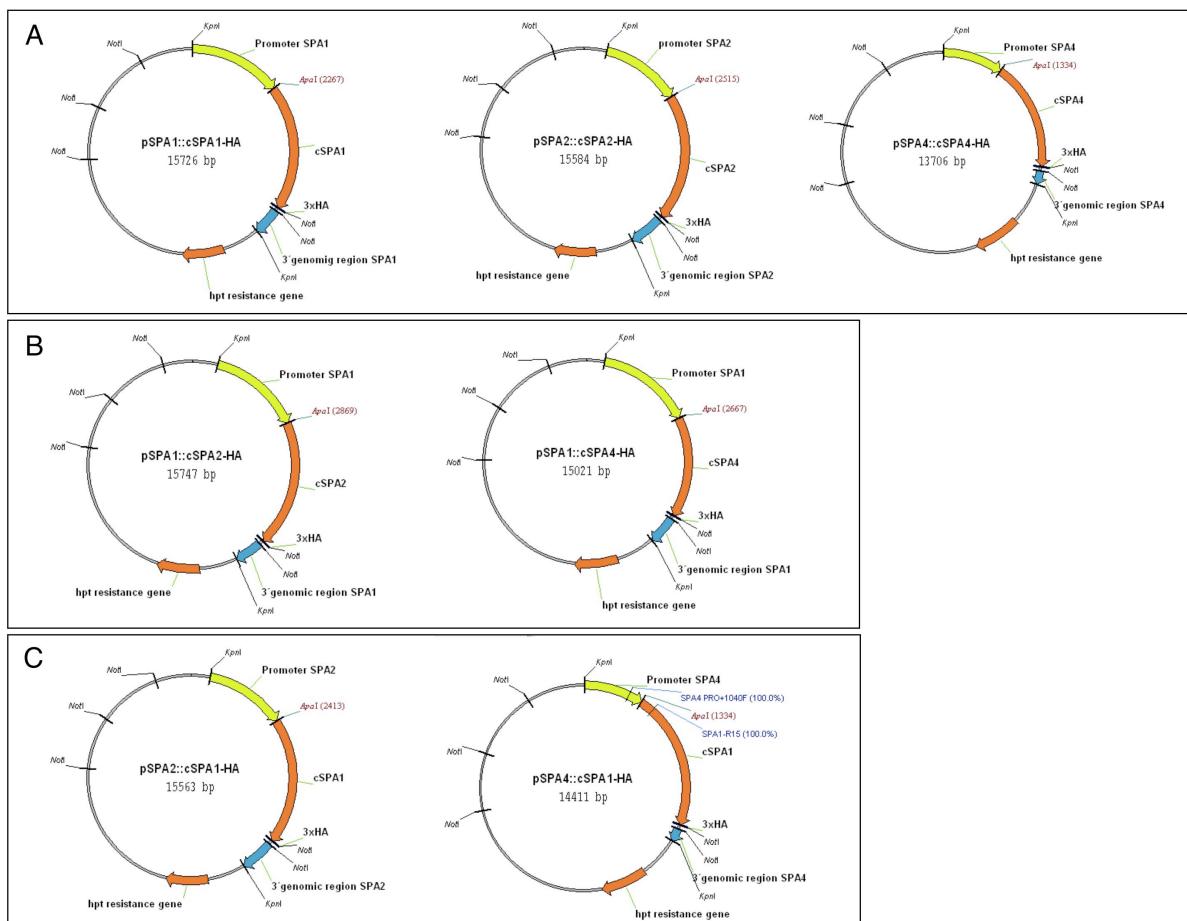

Figure 32: Maps of promoter-swap-constructs.

A: *SPA1*, *SPA2* and *SPA4* cDNAs under the regulation of its own endogenous 3´and5´regulatory sequences.
B: *cSPA2* and *cSPA4* under the control of *SPA1* regulatory elements.
C:*cSPA1* under the control of *SPA2* or *SPA4* regulatory sequences.
5' regulatory sequences are highlighted in green, 3' regulatory sequences in blue and coding sequences in orange.

The pJHA-212-hpt plasmid including *pSPA1::cSPA1-HA*, *pSPA2::cSPA2-HA* and *pSPA4::cSPA4-HA* were used for GUS fusion. With forward and reverse GUS

specific primers carrying both ApaI restriction sites GUS encoding sequence were amplified from pGWB3 without its Stop codon. After digestion with ApaI, GUS sequence without stop codon was ligated as a N-terminal SPA gene fusion (Figure 33).
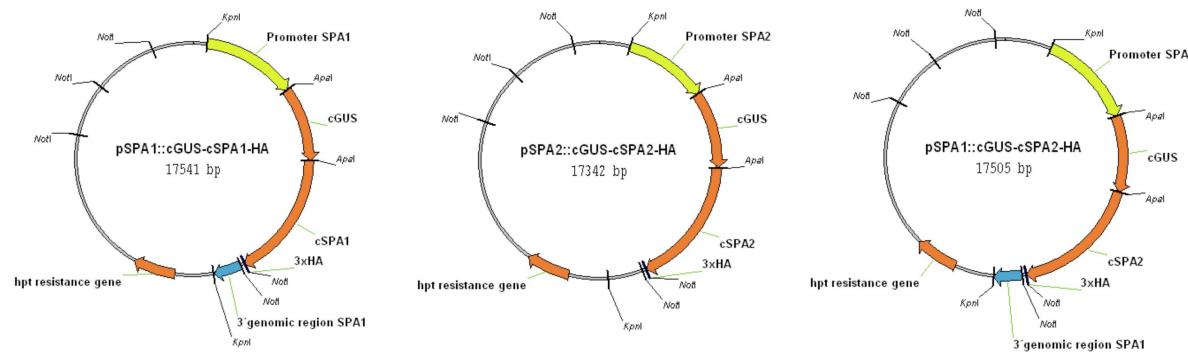

Figure 33: Constructs of GUS-SPA1-HA or GUS-SPA2-HA fusions controlled by *SPA1* or *SPA2* promoters.

5' regulatory sequences are highlighted in green, 3' regulatory sequences in blue and coding sequences in orange.

IV.3.2. *SPA promoter::GUS* constructs

SPA promoter::GUS constructs were generated by using gateway® cloning (Invitrogen). To create *pSPA1::GUS*, *pSPA2:: GUS* and *pSPA4::GUS* constructs 5`regulatory sequences of *SPA1*, *SPA2* and *SPA4* were amplified with primers introducing att-L recombination sites. The 5´regulatory sequence of *SPA1, SPA2* or *SPA4* was amplified with pSPA1-F and pSPA1-R, SPA2 pSPA2-F and pSPA2-R pSPA4-F and pSPA4-R, respectively. PCR products of *SPA1-*, *SPA2-* and *SPA4-* 5`regulatory sequences with flanking att-L1 and att-L2 sites were recombined into pDONR211 using BP clonase (Invitrogen). 5´regulatory sequences of *SPA1*, *SPA2* and *SPA4* were recombined from pDONR211 into pGWB3 with LR clonase (Invitrogen). pGWB3 is a promoter-less binary gateway destination vector with a gateway cassette before GUS coding sequence (Nakagawa et al., 2007). Plasmid clone that carries the 5´regulatory sequences of *SPA1*, *SPA2* or *SPA4* were selected for Agrobacterium GV3101-mediated transformation of wild-type plants (Clough and Bent, 1998). Transgenic plants were selected on MS medium supplemented with 50 mg/l kanamycin.
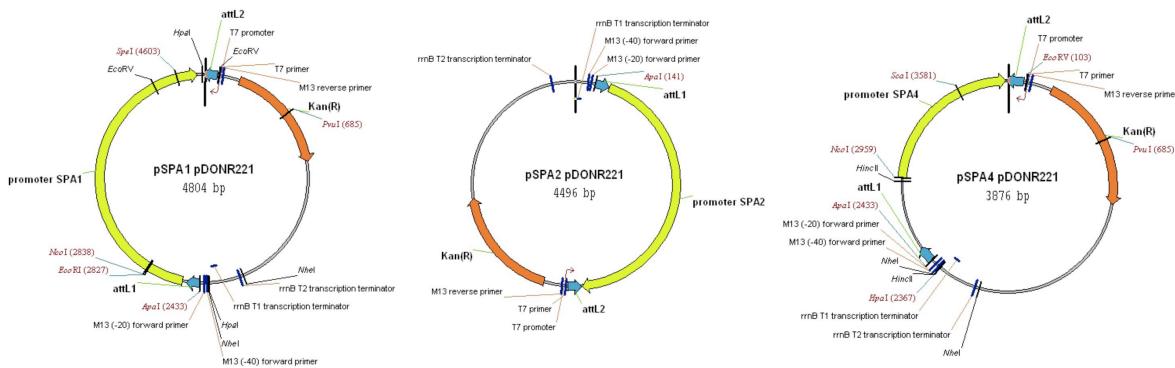
Figure 34: **Promoter of *SPA1*, *SPA2* and *SPA4* in pDONR221.**

5' regulatory sequences are highlighted in green and coding sequences in orange.
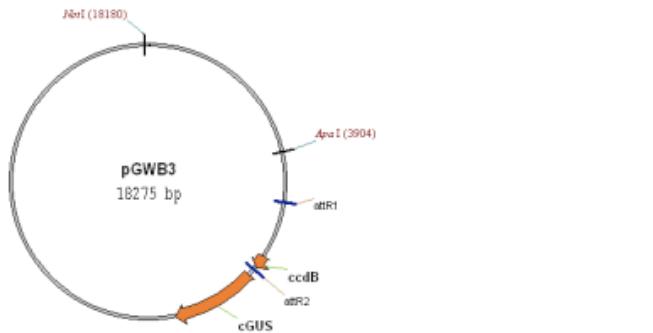
Figure 35: Gateway destination-vector pGWB3.

GUS coding sequence is highlighted in orange

IV.3.3. Construction of *SPA1::SPA1-HA* and *SPA1* deletion-constructs

To generate *SPA1::SPA1-HA*, a number of cloning steps were performed. First, a *SPA1* genomic fragment comprising 2241 bp of the 5´ sequence of *SPA1* and the *SPA1* gene up to, but not including, the Stop codon was amplified from a cosmid library (Rodriguez et al., 1998) using primers containing restriction recognition sites (SalI-XmaI for the F-primer and NotI for the R-primer). The (partially) digested PCR product was ligated into the SalI/NotI sites of pBS to generate SPA1-A-pBS. Subsequently, 1026 bp of the putative 3´-UTR of SPA1 were PCR-amplified using primers containing NotI or XmaI-SacI sites in the F- or R-primer, respectively. This PCR product was digested and ligated into NotI/SacI sites of SPA1-A-pBS to generate SPA1-B-pBS. Next, a sequence encoding the triple-HA-tag followed by a stop codon was amplified from a plasmid (Sato and Wada, 1997) by PCR using primers that both contain NotI sites, and the digested PCR product was subsequently

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cloned into the NotI site of SPA1-B-pBS to generate SPA1-C-pBS. The correct sequence of this construct was confirmed by sequencing. At last, the insert of SPA1-C-pBS was ligated into the XmaI site of pPZP211 (Hajdukiewicz et al., 1994) and a plasmid clone that carries the *SPA1* gene and the *35S-Kan* gene in the same orientations was selected for Agrobacterium GV3101-mediated transformation of *spa1-3* mutant plants (Clough and Bent, 1998). Transgenic plants were selected on MS medium supplemented with 50 mg/l kanamycin. When generating FL-SPA1 as the progenitor construct for the SPA1 structure/function analysis, the introns within the *SPA1* coding region were removed from SPA1-C-pBS in order to facilitate the construction of deletions. For this purpose, an AvrII-BamHI fragment from SPA1-C-pBS was replaced with a fragment from a *SPA1* cDNA clone. Hence, the construct FL-SPA1 carries the open-reading-frame (ORF) of *SPA1* under the control of *SPA1* 5´- and 3´-controlling sequences including the intron in the 5`-UTR. To generate ∆N (deletion of amino acid 24-478 of the wild-type SPA1 protein), the MfeI-SphI fragment comprising bp 51 until bp 1433 of the *SPA1* ORF was removed from FL-SPA1 by digestion and replaced by a double stranded oligo with MfeI and SphI sticky ends (MfeI-SphI-F and MfeI-SphI-R) To generate ∆KIN (deletion of amino acids 437-531 of the wild-type SPA1 protein), two PCR fragments were amplified from FL-SPA1 using the primer pairs SPA1-F and ∆KIN-R or ∆KIN-F and SPA1-R, respectively. Both PCR products were subsequently combined and used as templates to reamplify the ∆KIN deletion using the primers SPA1-F and SPA1-R. The thus obtained PCR product was digested with AvrII and PpuMI to replace the wild-type SPA1 sequence in the AvrII-PpuM1 double digested FL-SPA1 construct. To generate ∆cc (deletion of amino acids 566-639 of the wild-type SPA1 protein), two PCR fragments were amplified from FL-SPA1 using the primer pairs SPA1-F1 and ∆cc-R or ∆cc-F and SPA1-R, respectively. Both PCR products were subsequently combined and used as templates to reamplify the ∆cc deletion using the primers SPA1-F1 and SPA1-R. The thus obtained PCR product was digested with SphI and PpuMI to replace the wild-type SPA1 sequence in the SphI-PpuM1 double digested FL-SPA1 construct. For Agrobacterium-mediated transformation of plants, the inserts in FL-SPA1, ∆N, ∆KIN and ∆cc were subcloned into pPZP211 as described above for SPA1-C-pBS.
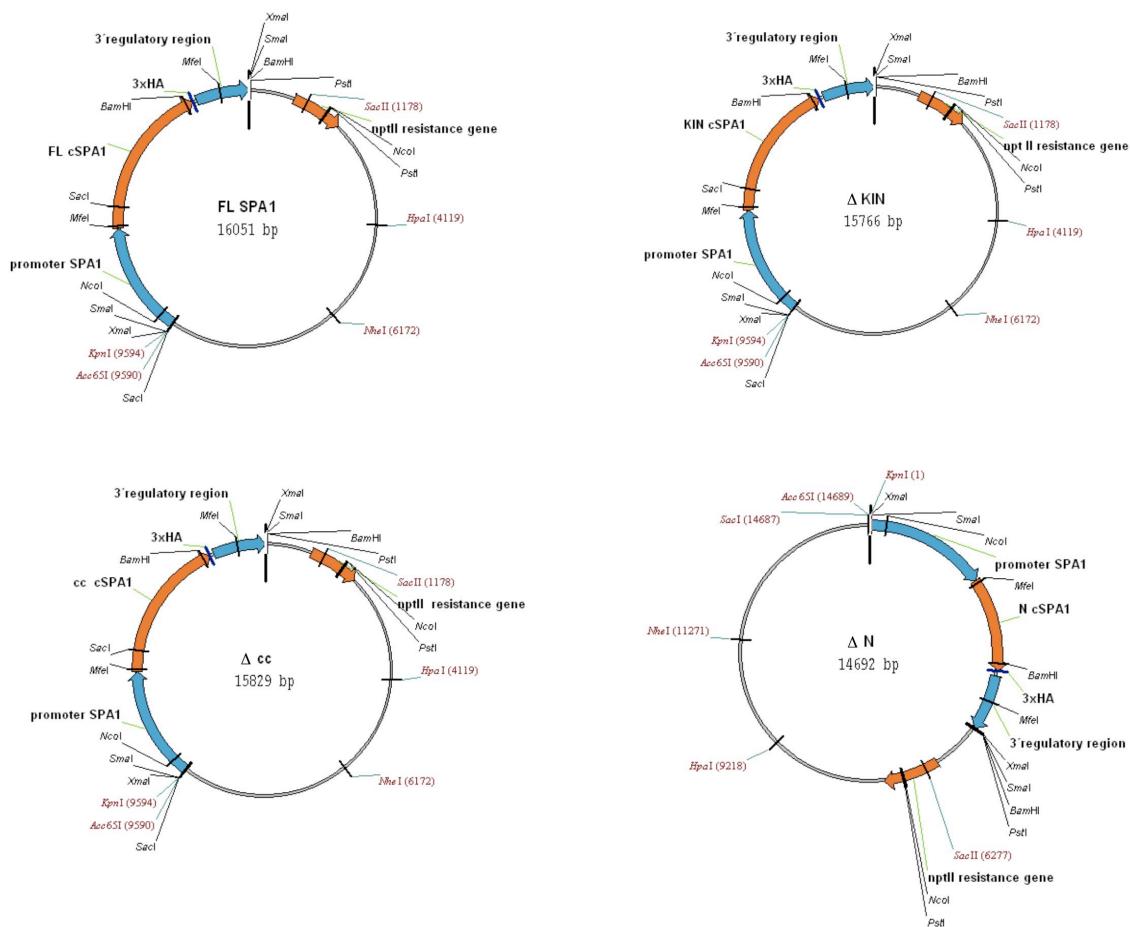
Figure 36: Constructs for SPA1 structure-function analysis.

5`and 3´regulatory sequences are highlighted in blue and coding sequence of *SPA1* and deletion-derivates are shown in orange.

V. Supplement
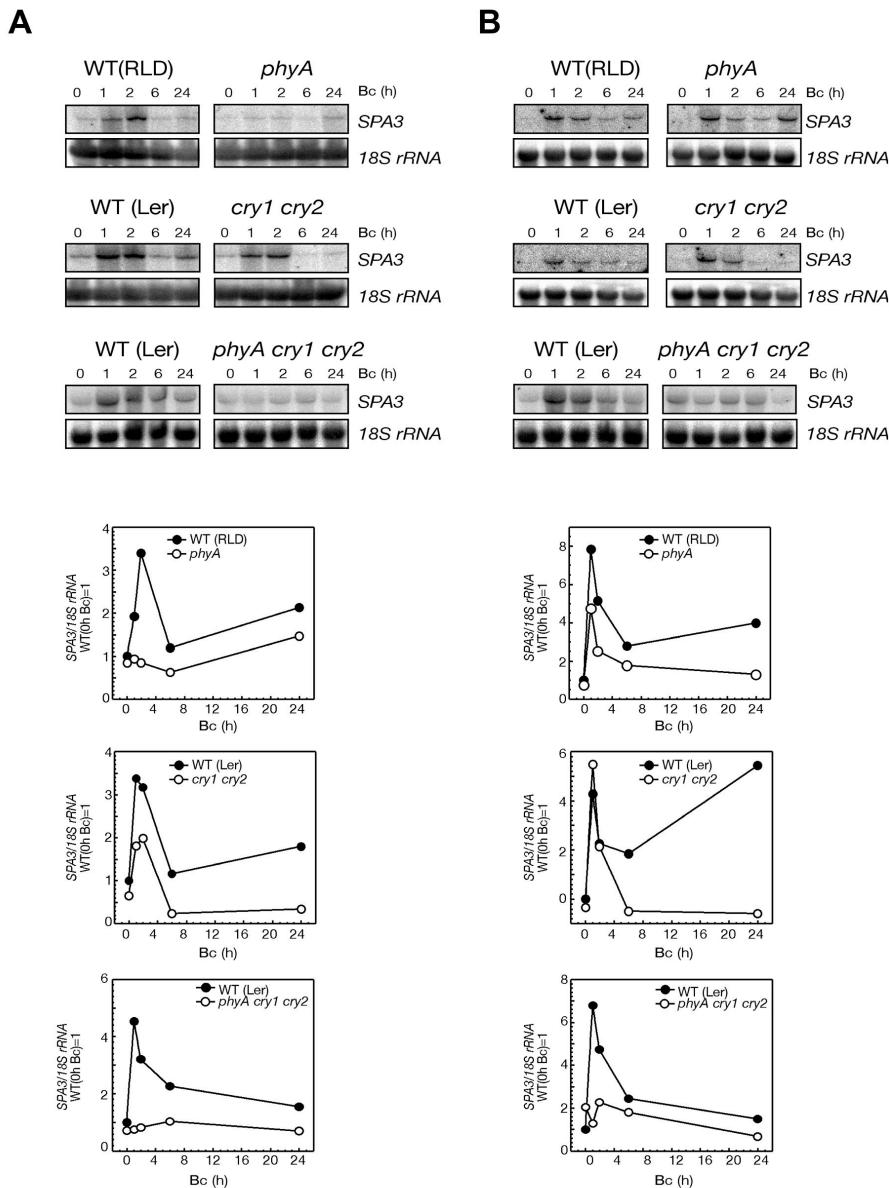
Figure 37: Bc-induced accumulation of *SPA3* transcripts requires phyA, cry1 and cry2 in a fluence dose dependent manner.

Total RNA gel blot analysis and quantification of *SPA3* transcript levels from wild-type (RLD/Ler) and *phyA* (RLD)*, cry1 cry2* (Ler)*, phyA cry1 cry2* (Ler) mutant seedlings that were transferred fromc-induced accumulation of *SPA3* transcripts requires phyA, cry1 and cry2 in a fluence dose dependent manner Total RNA gel blot analysis and quantification of *SPA3* transcript levels from wild-type (RLD/Ler) and *phyA* (RLD)*, cry1 cry2* (Ler)*, phyA cry1 cry2* (Ler) mutant seedlings that were transferred from darkness to 0.3 µ mol m^{-2} s^{-1} Bc **(A)** and to 5.0 µmol m^{-2} s^{-1} Bc **(B)** for 0-24 h.
SPA3 signals were normalized to *18S rRNA* levels after phosphorimager quantification.
A+B Total RNA gel blot analysis (left) and quantification (right) of SPA3 (A) and SPA4 (B) transcript levels in seedlings that were transferred from darkness to 5 µmol m^{-2} s^{-1} Bc for 0-24 h. Transcript levels were normalized by rehybridization with an *18SrRNA*-specific probe.
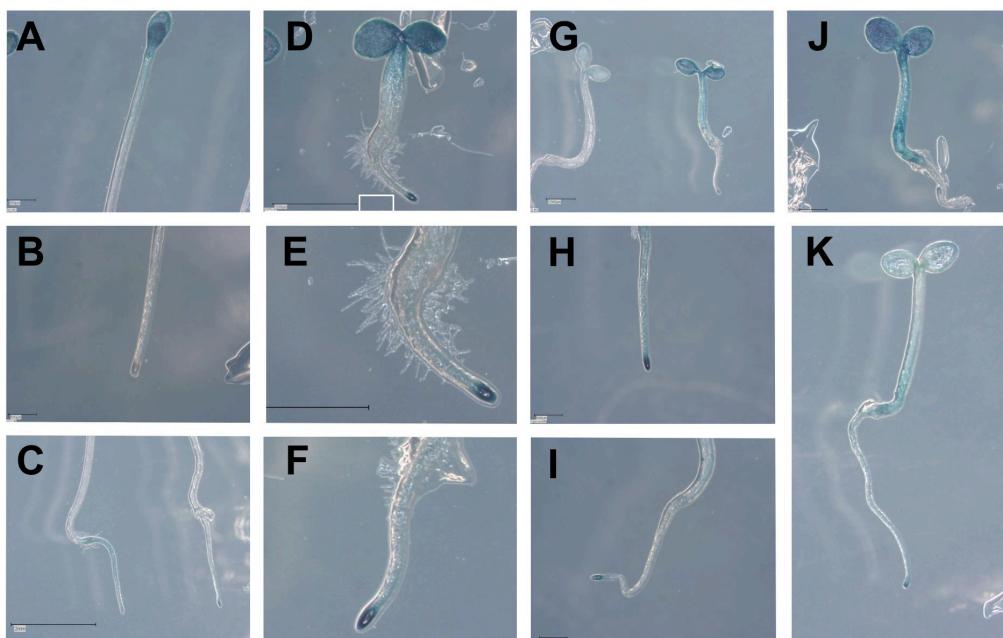
Figure 38: GUS-SPA2-HA and GUS-SPA1-HA are similar expresses in light

All shown seedlings are in segregating T2 generation. Those seedlings were grown for one day in darkness and for further three days in FR. After light treatment seedling were harvested under green light and directly transferred in GUS staining buffer. All following steps (vacuum infiltration and 37°C staining) are done in darkness. All seedlings were stained for 8 hours.

A, B, C: Cotyledons and part of the hypocotyl (A) and the roots (B and C) of transgenic *pPSA1::GUS-cSPA1-HA* seedlings in *spa1 spa2 spa3* mutant background .

D, E, F: Whole seedling (D) and roots (E) of mutants carrying *pSPA2::GUS-cSPA2-HA spa1 spa2 spa3 spa4* (D and E) or *spa1 spa2 spa3* (F) mutant background.

G, J, K: Whole seedlings expressing *pSPA1::GUS-cSPA2-HA* in either *spa1 spa2 spa3* triple mutant (G left one and K) or in segregating *spa1 spa2 spa3 spa4/SPA4* mutant background.

H, I: Roots of transgenic seedlings expressing *pSPA1::GUS-cSPA2-HA in spa1 spa2 spa3* mutant seedlings.
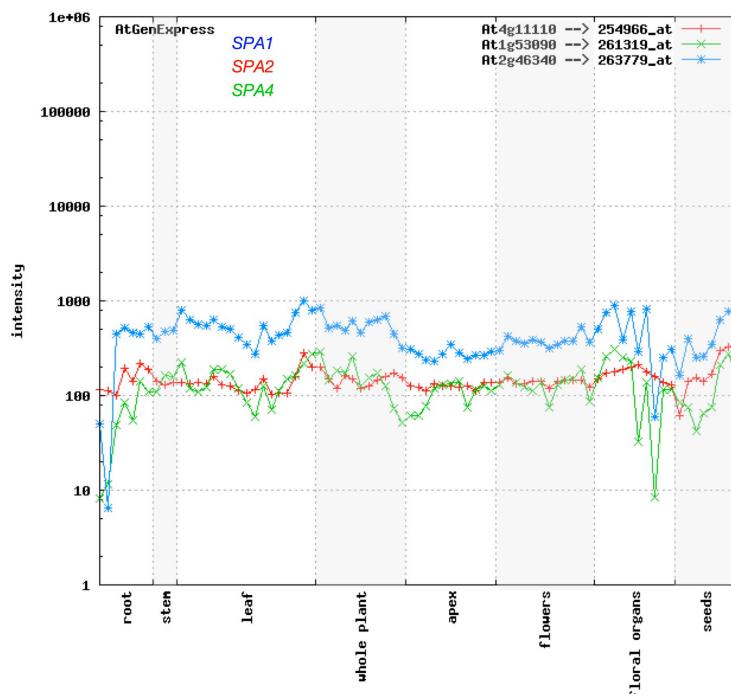
Figure 39: *SPA1*, *SPA2* and *SPA4* expression across different tissues and developmental stages.

Affymetrix microarray data from the AtGeneExpress are shown and extracted with the AtGeneExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp).
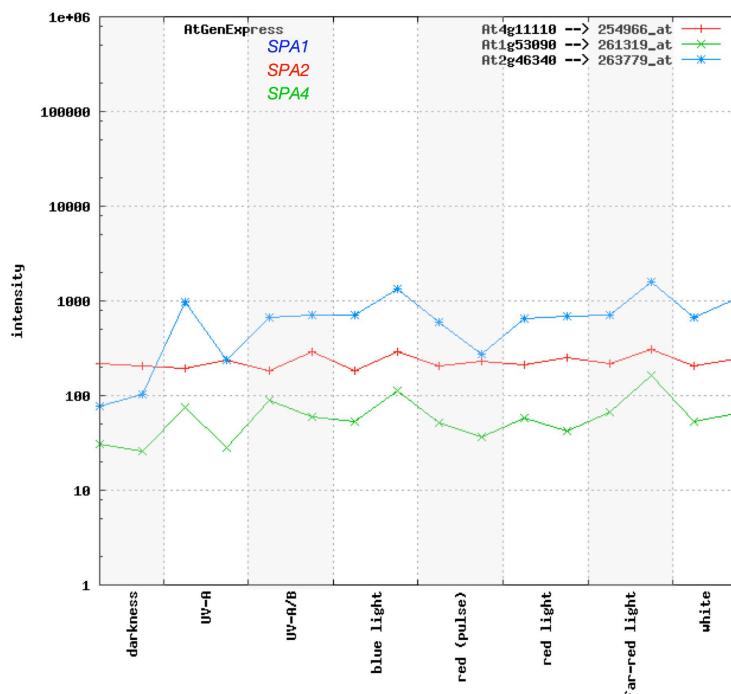
Figure 40: *SPA1*, *SPA2* and *SPA4* expression during different light treatments.

Affymetrix microarray data from the AtGeneExpress are shown and extracted with the AtGeneExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp).
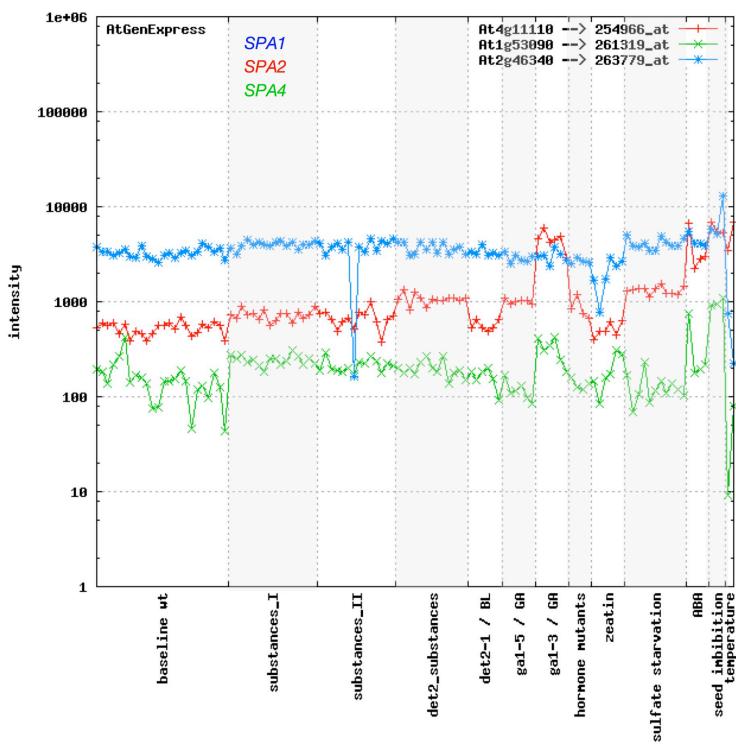
Figure 41: *SPA1*, *SPA2* and *SPA4* expression during different hormone treatments.

Affymetrix microarray data from the AtGeneExpress are shown and extracted with the AtGeneExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp).
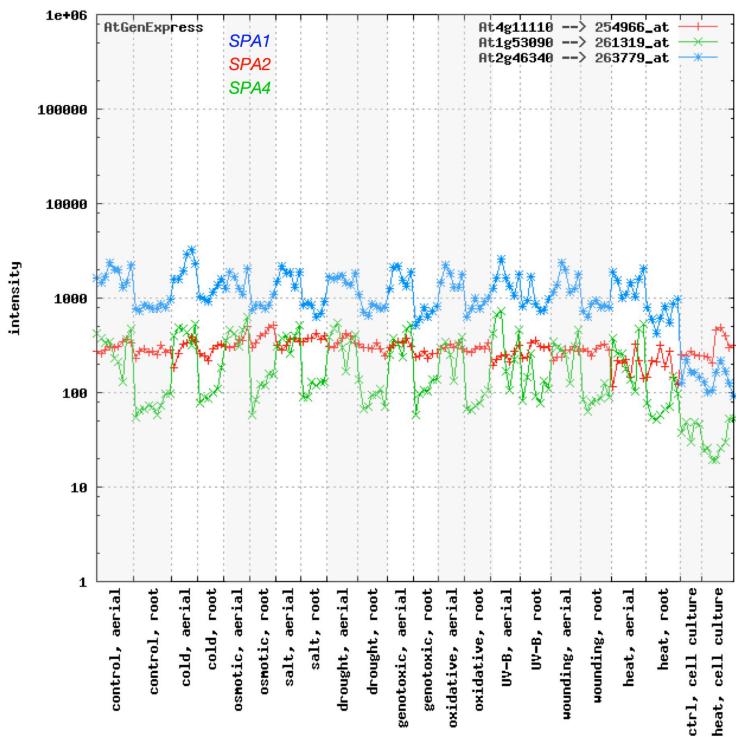
Figure 42: *SPA1*, *SPA2* and *SPA4* expression under abiotic stress conditions.

Affymetrix microarray data from the AtGeneExpress are shown and extracted with the AtGeneExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp).

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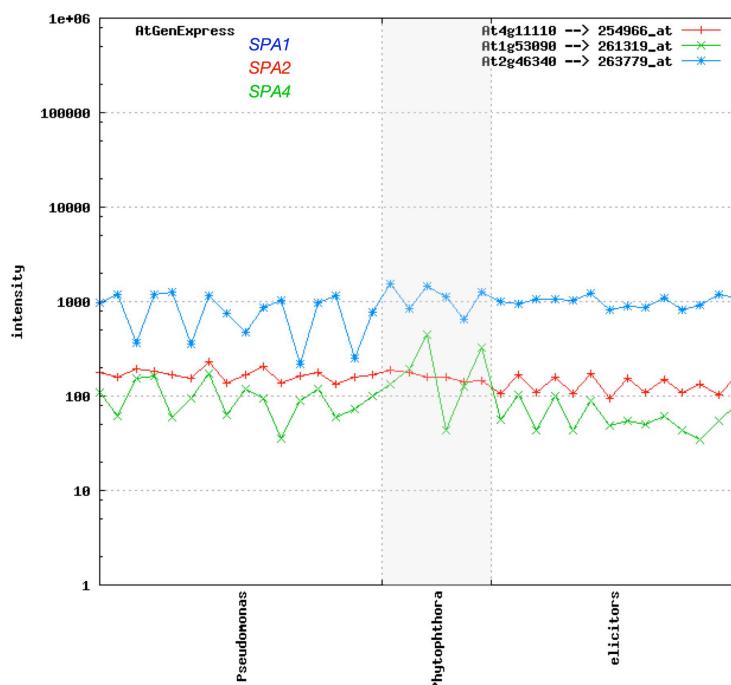
Figure 43: *SPA1*, *SPA2* and *SPA4* **expression under biotic stress conditions.**

Affymetrix microarray data from the AtGeneExpress are shown and extracted with the AtGeneExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp).

VI.   References

Ahmad, M., and Cashmore, A.R. (1993). *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. Nature **366,** 162-166.

Al-Sady, B., Kikis, E.A., Monte, E., and Quail, P.H. (2008). Mechanistic duality of transcription factor function in phytochrome signaling. Proc Natl Acad Sci U S A **105,** 2232-2237.

Al-Sady, B., Ni, W., Kircher, S., Schafer, E., and Quail, P.H. (2006). Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. Mol Cell **23,** 439-446.

An, H., Roussot, C., Suarez-Lopez, P., Corbesier, L., Vincent, C., Pineiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C., and Coupland, G. (2004). CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development **131,** 3615-3626.

Aukerman, M.J., Hirschfeld, M., Wester, L., Weaver, M., Clack, T., Amasino, R.M., and Sharrock, R.A. (1997). A deletion in the *PHYD* gene of the Arabidopsis Wassilewskija ecotype defines a role for phytochrome D in red/far-red light sensing. Plant Cell **9,** 1317-1326.

Ballesteros, M.L., Bolle, C., Lois, L.M., Moore, J.M., Vielle-Calzada, J.P., Grossniklaus, U., and Chua, N.H. (2001). LAF1, a MYB transcription activator for phytochrome A signaling. Genes Dev **15,** 2613-2625.

Baumgardt, R.L., Oliverio, K.A., Casal, J.J., and Hoecker, U. (2002). SPA1, a component of phytochrome A signal transduction, regulates the light signaling current. Planta **215,** 745-753.

Briggs, W.R., and Olney, M.A. (2001). Photoreceptors in plant photomorphogenesis to date. Five phytochromes, two cryptochromes, one phototropin, and one superchrome. Plant Physiol **125,** 85-88.

Briggs, W.R., and Christie, J.M. (2002). Phototropins 1 and 2: versatile plant blue-light receptors. Trends Plant Sci **7,** 204-210.

Briggs, W.R., and Spudich, J.L. (2005). Handbook of photosensory receptors.

Casal, J.J. (2000). Phytochromes, cryptochromes, phototropin: photoreceptor interactions in plants. Photochem Photobiol **71,** 1-11.

Cashmore, A.R., Jarillo, J.A., Wu, Y.J., and Liu, D.M. (1999). Cryptochromes: Blue light receptors for plants and animals. Science **284,** 760-765.

Castillon, A., Shen, H., and Huq, E. (2007). Phytochrome Interacting Factors: central players in phytochrome-mediated light signaling networks. Trends Plant Sci **12,** 514-521.

Chattopadhyay, S., Ang, L.H., Puente, P., Deng, X.W., and Wei, N. (1998). Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. Plant Cell **10,** 673-683.

Chory, J., Peto, C., Feinbaum, R., Pratt, L., and Ausubel, F. (1989). *Arabidopsis thaliana* mutant that develops as a light-grown plant in the absence of light. Cell **58,** 991-999.

Christie, J.M., Reymond, P., Powell, G.K., Bernasconi, P., Raibekas, A.A., Liscum, E., and Briggs, W.R. (1998). *Arabidopsis* NPH1: A flavoprotein with the properties of a photoreceptor for phototropism. Science **282,** 1698-1701.

Clack, T., Mathews, S., and Sharrock, R.A. (1994). The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: The sequences and expression of *PHYD* and *PHYE*. Plant Mol Biol **25,** 413-427.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16,** 735-743.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science **316,** 1030-1033.

Coupland, G., Igeno, M.I., Simon, R., Schaffer, R., Murtas, G., Reeves, P., Robson, F., Pineiro, M., Costa, M., Lee, K., and Suarez-Lopez, P. (1998). The regulation of flowering time by daylength in Arabidopsis. Symp Soc Exp Biol **51,** 105-110.

Deng, X.-W., Caspar, T., and Quail, P.H. (1991). *cop*1: A regulatory locus involved in light-controlled development and gene expression in *Arabidopsis*. Genes Dev **5,** 1172-1182.

Deng, X.W., Matsui, M., Wei, N., Wagner, D., Chu, A.M., Feldmann, K.A., and Quail, P.H. (1992). COP1, an Arabidopsis regulatory gene, encodes a protein with both a zinc-binding motif and a G beta homologous domain. Cell **71,** 791-801.

Devlin, P.F., and Kay, S.A. (2000). Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. Plant Cell **12,** 2499-2510.

Duek, P.D., and Fankhauser, C. (2003). HFR1, a putative bHLH transcription factor, mediates both phytochrome A and cryptochrome signalling. Plant J **34,** 827-836.

Duek, P.D., Elmer, M.V., van Oosten, V.R., and Fankhauser, C. (2004). The degradation of HFR1, a putative bHLH class transcription factor involved in light signaling, is regulated by phosphorylation and requires COP1. Curr Biol **14,** 2296-2301.

Fairchild, C.D., Schumaker, M.A., and Quail, P.H. (2000). HFR1 encodes an atypical bHLH protein that acts in phytochrome A signal transduction. Genes Dev **14,** 2377-2391.

Fittinghoff, K., Laubinger, S., Nixdorf, M., Fackendahl, P., Baumgardt, R.L., Batschauer, A., and Hoecker, U. (2006). Functional and expression analysis of Arabidopsis *SPA* genes during seedling photomorphogenesis and adult growth. Plant J **47,** 577-590.

Guo, H., Yang, H., Mockler, T.C., and Lin, C. (1998). Regulation of flowering time by Arabidopsis photoreceptors. Science **279,** 1360-1363.

Guo, H., Duong, H., Ma, N., and Lin, C. (1999). The Arabidopsis blue light receptor cryptochrome 2 is a nuclear protein regulated by a blue light-dependent post-transcriptional mechanism. Plant J **19,** 279-287.

Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994). The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol **25,** 989-994.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science **290,** 2110-2113.

Hisada, A., Hanzawa, H., Weller, J.L., Nagatani, A., Reid, J.B., and Furuya, M. (2000). Light-induced nuclear translocation of endogenous pea phytochrome A visualized by immunocytochemical procedures. Plant Cell **12,** 1063-1078.

Hoecker, U. (2005). Regulated proteolysis in light signaling. Curr Opin Plant Biol **8,** 469-476.

Hoecker, U., and Quail, P.H. (2001). The phytochrome A-specific signaling intermediate SPA1 interacts directly with COP1, a constitutive repressor of light signaling in Arabidopsis. J Biol Chem **276,** 38173-38178.

Hoecker, U., Xu, Y., and Quail, P.H. (1998). *SPA1*: A new genetic locus involved in phytochrome A - specific signal transduction. Plant Cell **10,** 19-33.

Hoecker, U., Tepperman, J.M., and Quail, P.H. (1999). SPA1, a WD-repeat protein specific to phytochrome A signal transduction. Science **284,** 496-499.

Huala, E., Oeller, P.W., Liscum, E., Han, I.S., Larsen, E., and Briggs, W.R. (1997). Arabidopsis NPH1: A protein kinase with a putative redox-sensing domain. Science **278,** 2120-2123.

Imaizumi, T., Tran, H.G., Swartz, T.E., Briggs, W.R., and Kay, S.A. (2003). FKF1 is essential for photoperiodic-specific light signalling in Arabidopsis. Nature **426,** 302-306.

Ishikawa, M., Kiba, T., and Chua, N.H. (2006). The Arabidopsis SPA1 gene is required for circadian clock function and photoperiodic flowering. Plant J **46,** 736-746.

Jaeger, K.E., and Wigge, P.A. (2007). FT protein acts as a long-range signal in Arabidopsis. Curr Biol **17,** 1050-1054.

Jang, I.C., Yang, J.Y., Seo, H.S., and Chua, N.H. (2005). HFR1 is targeted by COP1 E3 ligase for post-translational proteolysis during phytochrome A signaling. Genes Dev **19,** 593-602.

Jang, S., Marchal, V., Panigrahi, K.C., Wenkel, S., Soppe, W., Deng, X.W., Valverde, F., and Coupland, G. (2008). Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. Embo J **27,** 1277-1288.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. Embo J **6,** 3901-3907.

Kircher, S., Kozma-Bognar, L., Kim, L., Adam, E., Harter, K., Schäfer, E., and Nagy, F. (1999). Light quality-dependent nuclear import of the plant photoreceptors phytochrome A and B. Plant Cell **11,** 1445-1456.

Kleine, T., Lockhart, P., and Batschauer, A. (2003). An Arabidopsis protein closely related to Synechocystis cryptochrome is targeted to organelles. Plant J **35,** 93-103.

Kleiner, O., Kircher, S., Harter, K., and Batschauer, A. (1999). Nuclear localization of the Arabidopsis blue light receptor cryptochrome 2. Plant J **19,** 289-296.

Koornneef, M., Hanhart, C.J., and van der Veen, J.H. (1991). A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana. Mol Gen Genet **229,** 57-66.

Laubinger, S., and Hoecker, U. (2003). The SPA1-like proteins SPA3 and SPA4 repress photomorphogenesis in the light. Plant J **35,** 373-385.

Laubinger, S., Fittinghoff, K., and Hoecker, U. (2004). The SPA quartet: a family of WD-repeat proteins with a central role in suppression of photomorphogenesis in arabidopsis. Plant Cell **16,** 2293-2306.

Laubinger, S., Marchal, V., Gentilhomme, J., Wenkel, S., Adrian, J., Jang, S., Kulajta, C., Braun, H., Coupland, G., and Hoecker, U. (2006). Arabidopsis SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. Development **133,** 3213-3222.

Leivar, P., Monte, E., Oka, Y., Liu, T., Carle, C., Castillon, A., Huq, E., and Quail, P.H. (2008a). Multiple Phytochrome-Interacting bHLH Transcription Factors Repress Premature Seedling Photomorphogenesis in Darkness. Curr Biol.

Leivar, P., Monte, E., Al-Sady, B., Carle, C., Storer, A., Alonso, J.M., Ecker, J.R., and Quail, P.H. (2008b). The Arabidopsis phytochrome-interacting factor PIF7, together with PIF3 and PIF4, regulates responses to prolonged red light by modulating phyB levels. Plant Cell **20,** 337-352.

Lin, C., Yang, H., Guo, H., Mockler, T., Chen, J., and Cashmore, A.R. (1998). Enhancement of blue-light sensitivity of Arabidopsis seedlings by a blue light receptor cryptochrome 2. Proc Natl Acad Sci U S A **95,** 2686-2690.

Lin, C.T., Ahmad, M., and Cashmore, A.R. (1996). *Arabidopsis* cryptochrome 1 is a soluble protein mediating blue light-dependent regulation of plant growth and development. Plant J **10,** 893-902.

Lin, M.K., Belanger, H., Lee, Y.J., Varkonyi-Gasic, E., Taoka, K., Miura, E., Xoconostle-Cazares, B., Gendler, K., Jorgensen, R.A., Phinney, B., Lough, T.J., and Lucas, W.J. (2007a). FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. Plant Cell **19,** 1488-1506.

Lin, R., Ding, L., Casola, C., Ripoll, D.R., Feschotte, C., and Wang, H. (2007b). Transposase-derived transcription factors regulate light signaling in Arabidopsis. Science **318,** 1302-1305.

Liu, H., Yu, X., Li, K., Klejnot, J., Yang, H., Lisiero, D., and Lin, C. (2008). Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in Arabidopsis. Science **322,** 1535-1539.

Mas, P., Devlin, P.F., Panda, S., and Kay, S.A. (2000). Functional interaction of phytochrome B and cryptochrome 2. Nature **408,** 207-211.

Mathews, S., and Sharrock, R.A. (1997). Phytochrome gene diversity. Plant Cell Environ **20,** 666-671.

Mathieu, J., Warthmann, N., Kuttner, F., and Schmid, M. (2007). Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. Curr Biol **17,** 1055-1060.

Mazzella, M.A., Cerdan, P.D., Staneloni, R.J., and Casal, J.J. (2001). Hierarchical coupling of phytochromes and cryptochromes reconciles stability and light modulation of Arabidopsis development. Development **128,** 2291-2299.

Mockler, T., Yang, H., Yu, X., Parikh, D., Cheng, Y.C., Dolan, S., and Lin, C. (2003). Regulation of photoperiodic flowering by Arabidopsis photoreceptors. Proc Natl Acad Sci U S A **100,** 2140-2145.

Motchoulski, A., and Liscum, E. (1999). *Arabidopsis* NPH3: A NPH1 photoreceptor-interacting protein essential for phototropism. Science **286,** 961-964.

Nagatani, A., Reed, J.W., and Chory, J. (1993). Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. Plant Physiol **102,** 269-277.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T. (2007). Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng **104,** 34-41.

Ohgishi, M., Saji, K., Okada, K., and Sakai, T. (2004). Functional analysis of each blue light receptor, cry1, cry2, phot1, and phot2, by using combinatorial multiple mutants in Arabidopsis. Proc Natl Acad Sci U S A **101,** 2223-2228.

Onouchi, H., Igeno, M.I., Perilleux, C., Graves, K., and Coupland, G. (2000). Mutagenesis of plants overexpressing CONSTANS demonstrates novel interactions among Arabidopsis flowering-time genes. Plant Cell **12,** 885-900.

Osterlund, M.T., Wei, N., and Deng, X.W. (2000a). The roles of photoreceptor systems and the COP1-targeted destabilization of HY5 in light control of arabidopsis seedling development. Plant Physiol **124,** 1520-1524.

Osterlund, M.T., Hardtke, C.S., Wei, N., and Deng, X.W. (2000b). Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*. Nature **405,** 462-466.

Oyama, T., Shimura, Y., and Okada, K. (1997). The *Arabidopsis HY5* gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes Dev **11,** 2983-2995.

Para, A., Farre, E.M., Imaizumi, T., Pruneda-Paz, J.L., Harmon, F.G., and Kay, S.A. (2007). PRR3 Is a vascular regulator of TOC1 stability in the Arabidopsis circadian clock. Plant Cell **19,** 3462-3473.

Pedmale, U.V., and Liscum, E. (2007). Regulation of phototropic signaling in Arabidopsis via phosphorylation state changes in the phototropin 1-interacting protein NPH3. J Biol Chem **282,** 19992-20001.

Pepper, A., Delaney, T., Washburn, T., Poole, D., and Chory, J. (1994). DET1, a negative regulator of light-mediated development and gene expression in arabidopsis, encodes a novel nuclear-localized protein. Cell **78,** 109-116.

Putterill, J., Robson, F., Lee, K., Simon, R., and Coupland, G. (1995). The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell **80,** 847-857.

Quail, P.H. (1997). The phytochromes: A biochemical mechanism of signaling in sight? BioEssays **19,** 571-579.

Reed, J.W., Nagatani, A., Elich, T.D., Fagan, M., and Chory, J. (1994). Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. Plant Physiol **104,** 1139-1149.

Rodriguez, P.L., Benning, G., and Grill, E. (1998). ABI2, a second protein phosphatase 2C involved in abscisic acid signal transduction in Arabidopsis. FEBS Lett **421,** 185-190.

Saijo, Y., Sullivan, J.A., Wang, H., Yang, J., Shen, Y., Rubio, V., Ma, L., Hoecker, U., and Deng, X.W. (2003). The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. Genes Dev **17,** 2642-2647.

Saijo, Y., Zhu, D., Li, J., Rubio, V., Zhou, Z., Shen, Y., Hoecker, U., Wang, H., and Deng, X.W. (2008). Arabidopsis COP1/SPA1 complex and FHY1/FHY3 associate with distinct phosphorylated forms of phytochrome A in balancing light signaling. Mol Cell **31,** 607-613.

Sakamoto, K., and Briggs, W.R. (2002). Cellular and subcellular localization of phototropin 1. Plant Cell **14,** 1723-1735.

Sambrook, J., and Russell, D. (2001). Molecular Cloning: A Laboratory Manual.

Sato, M.H., and Wada, Y. (1997). Universal template plasmid for introduction of the triple-HA epitope sequence into cloned genes. Biotechniques **23,** 254-256.

Schepens, I., Duek, P., and Fankhauser, C. (2004). Phytochrome-mediated light signalling in Arabidopsis. Curr Opin Plant Biol **7,** 564-569.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Scholkopf, B., Weigel, D., and Lohmann, J.U. (2005). A gene expression map of Arabidopsis thaliana development. Nat Genet **37,** 501-506.

Schultz, T.F., Kiyosue, T., Yanovsky, M., Wada, M., and Kay, S.A. (2001). A role for LKP2 in the circadian clock of Arabidopsis. Plant Cell **13,** 2659-2670.

Schwechheimer, C., and Deng, X.W. (2000). The COP/DET/FUS proteins-regulators of eukaryotic growth and development. Semin Cell Dev Biol **11,** 495-503.

Seo, H.S., Watanabe, E., Tokutomi, S., Nagatani, A., and Chua, N.H. (2004). Photoreceptor ubiquitination by COP1 E3 ligase desensitizes phytochrome A signaling. Genes Dev **18,** 617-622.

Seo, H.S., Yang, J.Y., Ishikawa, M., Bolle, C., Ballesteros, M.L., and Chua, N.H. (2003). LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. Nature **424,** 995-999.

Shalitin, D., Yang, H.Y., Mockler, T.C., Maymon, M., Guo, H.W., Whitelam, G.C., and Lin, C.T. (2002). Regulation of Arabidopsis cryptochrome 2 by blue-light-dependent phosphorylation. Nature **417,** 763-767.

Sharrock, R.A., and Quail, P.H. (1989). Novel phytochrome sequences in *Arabidopsis thaliana*: Structure, evolution, and differential expression of a plant regulatory photoreceptor family. Genes Dev **3,** 1745-1757.

Shen, H., Zhu, L., Castillon, A., Majee, M., Downie, B., and Huq, E. (2008). Light-induced phosphorylation and degradation of the negative regulator PHYTOCHROME-INTERACTING FACTOR1 from Arabidopsis depend upon its direct physical interactions with photoactivated phytochromes. Plant Cell **20,** 1586-1602.

Shen, Y., Feng, S., Ma, L., Lin, R., Qu, L.J., Chen, Z., Wang, H., and Deng, X.W. (2005). Arabidopsis FHY1 protein stability is regulated by light via phytochrome A and 26S proteasome. Plant Physiol **139,** 1234-1243.

Somers, D.E., Kim, W.Y., and Geng, R. (2004). The F-box protein ZEITLUPE confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. Plant Cell **16,** 769-782.

Subramanian, C., Kim, B.H., Lyssenko, N.N., Xu, X., Johnson, C.H., and Von Arnim, A.G. (2004). The Arabidopsis repressor of light signaling, COP1, is regulated by nuclear exclusion: Mutational analysis by bioluminescence resonance energy transfer. Proc Natl Acad Sci U S A.

Sullivan, J.A., and Deng, X.W. (2003). From seed to seed: the role of photoreceptors in Arabidopsis development. Dev Biol **260,** 289-297.

Suzuki, G., Yanagawa, Y., Kwok, S.F., Matsui, M., and Deng, X.W. (2002). Arabidopsis COP10 is a ubiquitin-conjugating enzyme variant that acts together with COP1 and the COP9 signalosome in repressing photomorphogenesis. Genes Dev **16,** 554-559.

Ulm, R., Baumann, A., Oravecz, A., Mate, Z., Adam, E., Oakeley, E.J., Schafer, E., and Nagy, F. (2004). Genome-wide analysis of gene expression reveals function of the bZIP transcription factor HY5 in the UV-B response of Arabidopsis. Proc Natl Acad Sci U S A **101,** 1397-1402.

Usami, T., Mochizuki, N., Kondo, M., Nishimura, M., and Nagatani, A. (2004). Cryptochromes and phytochromes synergistically regulate Arabidopsis root greening under blue light. Plant Cell Physiol **45,** 1798-1808.

Von Arnim, A.G., and Deng, X.-W. (1994). Light inactivation of arabidopsis photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. Cell **79,** 1035-1045.

Von Arnim, A.G., Osterlund, M.T., Kwok, S.F., and Deng, X.W. (1997). Genetic and developmental control of nuclear accumulation of COP1, a repressor of photomorphogenesis in Arabidopsis. Plant Physiol **114,** 779-788.

Wang, H., and Deng, X.W. (2002). Arabidopsis FHY3 defines a key phytochrome A signaling component directly interacting with its homologous partner FAR1. Embo J **21,** 1339-1349.

Wang, H.Y., Ma, L.G., Li, J.M., Zhao, H.Y., and Deng, X.W. (2001). Direct interaction of Arabidopsis cryptochromes with COP1 in light control development. Science **294,** 154-158.

Wei, N., Kwok, S.F., Von Arnim, A.G., Lee, A., McNellis, T.W., Piekos, B., and Deng, X.-W. (1994). Arabidopsis *COP8, COP10*, and *COP11* genes are involved in repression of photomorphogenic development in darkness. Plant Cell **6,** 629-643.

Whitelam, G.C., Johnson, E., Peng, J., Carol, P., Anderson, M.L., Cowl, J.S., and Harberd, N.P. (1993). Phytochrome A null mutants of arabidopsis display a wild-type phenotype in white light. Plant Cell **5,** 757-768.

Yamaguchi, A., Kobayashi, Y., Goto, K., Abe, M., and Araki, T. (2005). TWIN SISTER OF FT (TSF) Acts as a Floral Pathway Integrator Redundantly with FT. Plant Cell Physiol **46,** 1175-1189.

Yanagawa, Y., Sullivan, J.A., Komatsu, S., Gusmaroli, G., Suzuki, G., Yin, J., Ishibashi, T., Saijo, Y., Rubio, V., Kimura, S., Wang, J., and Deng, X.W. (2004). Arabidopsis COP10 forms a complex with DDB1 and DET1 in vivo and enhances the activity of ubiquitin conjugating enzymes. Genes Dev **18,** 2172-2181.

Yang, H.Q., Tang, R.H., and Cashmore, A.R. (2001). The signaling mechanism of Arabidopsis CRY1 involves direct interaction with COP1. Plant Cell **13,** 2573-2587.

Yang, J., and Wang, H. (2006). The central coiled-coil domain and carboxyl-terminal WD-repeat domain of Arabidopsis SPA1 are responsible for mediating repression of light signaling. Plant J.

Yang, J., Lin, R., Hoecker, U., Liu, B., Xu, L., and Wang, H. (2005a). Repression of light signaling by Arabidopsis SPA1 involves post-translational regulation of HFR1 protein accumulation. Plant J **43,** 131-141.

Yang, J., Lin, R., Sullivan, J., Hoecker, U., Liu, B., Xu, L., Deng, X.W., and Wang, H. (2005b). Light Regulates COP1-Mediated Degradation of HFR1, a Transcription Factor Essential for Light Signaling in Arabidopsis. Plant Cell **17,** 804-821.

Yanovsky, M.J., Mazzella, M.A., Whitelam, G.C., and Casal, J.J. (2001). Resetting of the circadian clock by phytochromes and cryptochromes in Arabidopsis. J Biol Rhythms **16,** 523-530.

Yu, X., Klejnot, J., Zhao, X., Shalitin, D., Maymon, M., Yang, H., Lee, J., Liu, X., Lopez, J., and Lin, C. (2007). Arabidopsis cryptochrome 2 completes its posttranslational life cycle in the nucleus. Plant Cell **19,** 3146-3156.

Zhu, D., Maier, A., Lee, J.H., Laubinger, S., Saijo, Y., Wang, H., Qu, L.J., Hoecker, U., and Deng, X.W. (2008). Biochemical characterization of Arabidopsis complexes containing CONSTITUTIVELY PHOTOMORPHOGENIC1 and SUPPRESSOR OF PHYA proteins in light control of plant development. Plant Cell **20,** 2307-2323.

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VIII. Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit –einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde.

Die von mir vorgelegte Dissertation ist von Prof. Dr. Ute Höcker betreut worden.

Kirsten Fittinghoff

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Lebenslauf

Kirsten Fittinghoff
Johannastr.15
42549 Velbert
Geburtdatum: 19.12.1976
Geburtsort: Velbert

Schulausbildung
1983-1987   Gerhart-Hauptmann-Grundschule in Velbert
1987-1996   Nikolaus-Ehlen-Gymnasium in Velbert
            Abschluss: Allgemeine Hochschulreife

Hochschulausbildung
1996-1998   Grundstudium der Biologie an der Heinrich-Heine-Universität
            Düsseldorf
1999-2003   Hauptstudium der Biologie an der Heinrich-Heine-Universität Düsseldorf
            mit Schwerpunkt Botanik/Molekulare Entwicklungsbiologie, Biophysik und
            Organische Chemie
6/03-08/04 Diplomarbeit im Fach Botanik/ Molekulare Entwicklungsbiologie der Pflanze
            Theme: Molekulare Untersuchungen zur negativen Regulation der
            Lichtsignaltransduktion bei *Arabidopsis thaliana*
            Betreuerin: PD Dr. Ute Höcker, im Institut für Entwicklungs- und
            Molekularbiologie der Pflanzen, Universität Düsseldorf
Seit 01/2005 Promotion: „Funktionsanalyse der *SPA* Gen-Familie in *Arabidopsis thaliana*".
            Betreuerin: Prof. Dr. Ute Höcker

Teilnahme an wissenschaftlichen Konferenzen
- ausgewählter Sprecher
2007   Tagung „Molekularbiologie der Pflanzen" in Dabringhausen, Deutschland

- mit Posterpresentation
2006   "International Plant Photobiology Meeting" in Paris, Frankreich
2007   EPS Summerschool: "Environmental signalling: Arabidopsis as a model" in
            Utrecht, Niederlande

- weitere Konferenzteilnahmen:
2003:   „International Plant Photobiology Meeting" in Marburg, Deutschland
2002:   „Deutsche Botanikertagung" in Freiburg, Deutschland

Publikationen

Fittinghoff, K., Laubinger, S., Nixdorf, M., Fackendahl, P., Baumgardt, R.L., Batschauer, A., and Hoecker, U. (2006).
    Functional and expression analysis of *Arabidopsis* SPA genes during seedling
    photomorphogenesis and adult growth.
*The Plant Journal **47,** 577-590.*

Laubinger, S., Fittinghoff, K., and Hoecker, U. (2004).
    The SPA quartet: a family of WD-repeat proteins with a central role in suppression of
    photomorphogenesis in *Arabidopsis*.
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