Functional Analysis of the SPA Gene Family in Arabidopsis thaliana

Inaugural-Dissertation

zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von

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2008
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Tag der mündlichen Prüfung: 02.02.2009
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### Abbreviations

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

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

**Nomenclature:**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>SPA1</td>
<td>gene, locus, wild-type allele</td>
</tr>
<tr>
<td>spa1</td>
<td>mutant allele</td>
</tr>
<tr>
<td>SPA1 protein</td>
<td>protein</td>
</tr>
</tbody>
</table>

**Exception:** photoreceptors

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>PHY</td>
<td>gene, locus, wild-type allele</td>
</tr>
<tr>
<td>phy</td>
<td>mutant allele</td>
</tr>
<tr>
<td>PHY</td>
<td>apoprotein (without chromophor)</td>
</tr>
<tr>
<td>phy</td>
<td>holoprotein (with chromophor)</td>
</tr>
</tbody>
</table>
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.

Zusammenfassung


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...
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, phy 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? phy 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 phy 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 **CONSTITUTIVE PHOTOMORPHOGENESIS** (COP), **DE-ETIOLATED** (DET) and **FUSCA** (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
Introduction

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 cop1bid6 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.](image)

**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 *FLOWERING LOCUS T* (*FT*) and its homolog *TWIN-SISTER OF FT* (*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

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**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 proteasome. 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): \( \Delta N \) lacks most
of the SPA1 N-terminus, ∆KIN 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 (∆cc; 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 8A) 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 8A). Therefore, transgenic spa1-3 seedlings were analyzed under fluence rate of FRc.

The vast majority of all investigated transgenic T2 lines carrying FL SPA1, ∆N or ∆KIN deletion-derivates of SPA1 showed segregated seedlings with long hypocotyls and partially closed cotyledons in low FRc, like WT seedlings (Figure 8B). Thus, expression of FL SPA1 or either its deletion-derivates ∆KIN or ∆N in spa1-3 mutants fully restored the WT phenotype (Figure 8B). 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, ∆KIN or ∆N 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
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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 (KKKKKASK) 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
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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 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, ΔN or ΔKIN 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), ΔKIN or ΔN. 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).
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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 18S rRNA-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 18S rRNA-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 rehybridization 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](image-url): 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.

![Image of RNA gel blot analysis](image)

**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.](image)

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.

![Image of GUS staining in leaves](image)

**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.
Results

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 aspiring. 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.

**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 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.](image)

**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).
Results

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 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).
Results

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 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
Results

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*. 
Results

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.

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<tr>
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<tr>
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<tr>
<td>pSPA4::cSPA1-HA</td>
<td>partial complementation</td>
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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.
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-HA or SPA2-HA under the control of their endogenous promoter SPA1 and SPA2. For SPA2 cDNA a further fusion with GUS is expressed by 5’ and 3’ 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/deltfus 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 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.
Figure 30: Possible light-dependent 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
Discussion

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
\(\alpha\)-\([{}^{32}\text{P}]\text{-dATP}\) 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, \(\alpha\) HA antibody from Roche (Mannheim, Germany) and \(\alpha\) Tubulin from Sigma-Aldrich (Deisenhofen, Germany) were used for immunodetection.

IV.1.3. Bacterial strains
For standard cloning, *Escherichia coli* strain DH5\(\alpha\) 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**

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## Materials and Methods

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### Footnotes

- ApaI
- Sacl
- KpnI
- NotI
- SacI
- Stop
### Materials and Methods

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<td>distinguish cSPA4 from gSPA4</td>
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<tr>
<td>4-F7</td>
<td>GTAACCTTGAAGGCGGTGGTCAG</td>
<td>distinguish cSPA4 from gSPA4</td>
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</tbody>
</table>

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, 32P-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 β-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 NaPO₄ (pH 7.0), 0.5 mM potassium-ferrocyanide (K₄Fe(CN)₆+ H₂O), 0.5 mM potassium ferricyanide (K₃Fe(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 \textit{SPA1}, \textit{SPA2} and \textit{SPA4} were amplified from genomic DNA (Col) with primers introducing a 5´ KpnI and a 3´ Apal restriction site (SPA1: -2260 bp; SPA2: -1958 bp and SPA4: -1309 bp before ATG; Table 1). Those PCR products were digested with KpnI and Apal and ligated into pBluescript (pBS KS; Stratagene). pBS plasmids carrying 5´ regulatory sequences of \textit{SPA1}, \textit{SPA2} or \textit{SPA4} (\textit{SPA1}, \textit{SPA2} or \textit{SPA4} promoters) were sequenced.

Second, 3´ regulatory sequences of \textit{SPA1}, \textit{SPA2} and \textit{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 \textit{SPA1}+ 672 bp after Stop, \textit{SPA2} +812 bp after Stop, \textit{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 \textit{SPA1}, \textit{SPA2} and \textit{SPA4}, respectively. Resulting pBS vectors carrying \textit{SPA}x 5´ and \textit{SPA}x 3´ regulatory sequences were used for further cloning.

Complementary DNAs (cDNAs) of \textit{SPA1}, \textit{SPA2} and \textit{SPA4} were amplified from previous described constructs: \textit{cSPA1} derived from amplification with FL \textit{SPA1} used as a template (Fittinghoff et al. 2006). \textit{cSPA2} was amplified from 35S::GUS-cSPA2 (Laubinger et al., 2004). \textit{SPA4} cDNA was derived from PCR amplification with cSPA4-TOPO as a template (kindly provided by Ute Hoecker). \textit{SPA} cDNAs were amplified without their stop codons with forward primers introducing a 3´ Apal 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, \textit{SPA} cDNAs without stop codons were digested with Apal and NotI and ligated into the Apal and NotI site of pBs contain the \textit{SPA} 5´ and 3´ regulatory sequences resulting in following constructs \textit{pSPA1::{cSPA1}}, \textit{pSPA2::{cSPA2}}, \textit{pSPA4::{cSPA4}}, \textit{pSPA1::{cSPA2}}, \textit{pSPA1::{cSPA4}}, \textit{pSPA2::{cSPA1}} and \textit{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.](image)

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

![Figure 33](image)

**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.
IV.3.3. Construction of \textit{SPA1::SPA1-HA} and \textit{SPA1} deletion-constructs

To generate \textit{SPA1::SPA1-HA}, a number of cloning steps were performed. First, a \textit{SPA1} genomic fragment comprising 2241 bp of the 5' sequence of \textit{SPA1} and the \textit{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-Xmal 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 \textit{SPA1} were PCR-amplified using primers containing NotI or Xmal-Sacl sites in the F- or R-primer, respectively. This PCR product was digested and ligated into NotI/Sacl 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 Xmal 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-BamHl 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 ΔN (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 ΔKIN (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 ΔKIN-R or ΔKIN-F and SPA1-R, respectively. Both PCR products were subsequently combined and used as templates to reamplify the ΔKIN 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 Δcc (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 Δcc-R or Δcc-F and SPA1-R, respectively. Both PCR products were subsequently combined and used as templates to reamplify the Δcc 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, ΔN, ΔKIN and Δcc 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 from dark-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 μmol 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).
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


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VII. Danksagung

Zuerst möchte ich Frau Prof. Dr. Ute Höcker danken für die Betreuung und die Möglichkeit selbständig zu arbeiten. Zudem danke ich Ihr für alles was ich von Ihr lernen konnte. Herrn Prof. Dr. Hülskamp danke ich für die Übernahme des Zweitgutachtens. Ich danke Herrn Prof. Dr. Werr für die Übernahme des Prüfungsvorsitzes. Ich hatte das Privileg die erste Hälfte meiner Doktorarbeit in dem Institut von Herrn Prof. Dr. Westhoff durchzuführen. Ich danke Ihm und seinen Mitarbeiter(inne)n für konstruktive Besprechungen und vor allem für die herzliche und familiäre Atmosphäre. Dafür danke ich: Karin, Udo, Susanne, Nino, Elena, Drea, Dagmar, Sabine, Monika, Maria, Sascha und Kerstin.


Ich möchte besonders Gabi Fiene danken, die mir immer mit Rat und Tat zur Seite steht und alles möglich macht. Petra Fackendahl und Alex Maier danke ich für die schöne gemeinsame Zeit und die viele Unterstützung. Thank you Aashish for being such a good colleague and friend. Ich danke Sylvia Glatzel für die moralische Unterstützung während des Schreibens und für wahnwitzige Momente mit Ihrem Betreuer. Ich danke der ganzen AG Höcker für die Unterstützung und das tolle Arbeitsklima. Ich danke zu dem Anke Hüser für Ihre grenzenlose Hilfsbereitschaft.

Ich danke Lindy Baumgardt für Ihre Freundschaft und ihr Verständnis.

Aber der größte Dank gilt meiner Familie: meinen Eltern Herbert und Helga Fittinghoff, meinen Geschwistern Christine und Dirk, meinem Schwager Uwe, meinen Neffen Tim und Marc und meinem Lebenspartner Sascha Laubinger. Ich danke Euch für die gemeinsame Unterstützung, für den Zusammenhalt; das Vertrauen und für die Verlässlichkeit.
VIII. Erklärung


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

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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

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

Publikationen
