The role of COP1/SPA in light signaling: Growth control, cell-cell communication and functional conservation in plants

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Abbreviations

35S	*35S* promoter of Cauliflower Mosaic viruc
BR	Brassinosteroids
B	blue light
°C	degree Celsius
µl	micro litre
µg	micro gram
bp	base pair
bHLH	basic helix-loop-helix
cDNA	complementary DNA
CC	coiled-coil structure
Col	Columbia; ecotype of *Arabidopsis thaliana*
cm	centimetre
D	darkness
Da	Dalton
DNA	deoxyribonucleic acid
FR	far-red light
FRc	continuous FR
GUS	β-Glucuronidase
h	hour
kb	kilo bp
kDA	kilo Da
l	litre
LB	T-DNA left border
LD	long day
M	molar; mol/l
mg	milligram
mM	millimolar
min	minute
mRNA	messenger-ribonucleic-acid
MS	Murashige and Skoog medium
NLS	nuclear localization signal/sequence
ORF	open reading frame
PCR	polymerase chain reaction
Pfr	red light absorbing phytochrome conformation
Phy	phytochrome

Pr	red light absorbing phytochrome conformation
R	red light
RB	T-DNA right border
RLD	ecotype of *Arabidopsis thaliana*
RNA	ribonucleic acid
RT-PCR	reverse-transcription-PCR
s	second
SD	short day
UV	ultraviolet
WD	aspartic acid; tryptophan
WT	wild type

Gene and protein nomenclature:

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

Exception: photoreceptors

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

Abstract

Light is one of the most important environmental factors affecting almost all stages of plant growth and development. Arabidopsis SPA and COP1 proteins act as repressors of light signaling in darkness. Members of the SPA protein family (SPA1-SPA4) can physically interact with COP1 and, together, they constitute a functional E3-ubiquitin ligase complex. The COP1/SPA complex regulates seedling development, stomata differentiation, leaf size and photoperiodic flowering in Arabidopsis by targeting transcription factors such as HY5, HFR1, CO etc. for degradation.

In the present study, I investigated in which tissues SPA1 needs to be expressed to regulate different plant developmental processes. To this end, I expressed a GUS-SPA1 fusion protein under the control of various tissue-specific promoters (phloem, leaf-mesophyll, epidermis, meristem and root) in a *spa* mutant background and analyzed the transgenic plants for complementation of the *spa* mutant phenotype. The results show that SPA1 functions exclusively in the phloem to regulate photoperiodic flowering suggesting that SPA1 acts cell-autonomously in the phloem to target its substrate CO for degradation. To regulate the leaf size, SPA1 acts in both the phloem and the leaf mesophyll, but not in the epidermis indicating non-cell autonomous effects in SPA1-dependent leaf size regulation. Moreover, phloem-specific expression of SPA1 has major effects on seedling development in both darkness and light. Eventually, stomata differentiation and epidermal pavement cell shape are also regulated by phloem-specific functions of SPA1. These results indicate that cell-cell communication plays a very important role in SPA1-regulated plant developmental processes.

SPA proteins and, therefore, the COP1/SPA complexes are plant specific. However, the function of COP1 and SPA proteins are not known in plant species other than the dicot Arabidopsis. In a second project, I examined the functionality of the COP1 and SPA proteins from the moss Physcomitrella and the monocot rice in Arabidopsis. To this end, I expressed the open reading frames of rice and Physcomitrella *COP1* and *SPA* homologs in Arabidopsis *cop1* and *spa* mutant plants, respectively, and then analyzed the transgenic plants for complementation of the respective mutant phenotypes. Rice and Physcomitrella *COP1* homologs were functional in Arabidopsis, whereas *SPA* homologs from these species were not functional, suggesting a conserved basic mechanism of action of COP1, but functional divergence of SPA proteins during plant evolution. Interestingly, Physcomitrella COP1 and SPA proteins interact in vitro suggesting the possibility of formation of a COP1/SPA complex early in evolution.

Zusammenfassung

Licht beeinflusst nahezu alle Phasen der pflanzlichen Entwicklung. Arabidopsis SPA- und COP1-Proteine repräsentieren zentrale Komponenten der Lichtsignaltransduktion, da sie als Repressoren der Lichtantwort in Dunkelheit fungieren. Die Mitglieder der SPA-Proteinfamilie (SPA1-SPA4) interagieren mit COP1 und bilden zusammen einen funktionellen E3-Ubiquitinligase-Komplex. Dieser COP1/SPA-Komplex reguliert die Keimlingsentwicklung, die Differenzierung der Spaltöffnungen, die Blattgröße und die photoperiodische Induktion der Blütenbildung in Arabidopsis, indem er Transkriptionsfaktoren wie HY5, HFR1 und CO ubiquitiniert und dadurch ihre Degradation herbeiführt.

Ziel der vorliegenden Arbeit war es, festzustellen, in welchen Geweben SPA1 bestimmte pflanzliche Entwicklungsprozesse reguliert. Hierzu wurde ein GUS-SPA1-Fusionsprotein unter der Kontrolle verschiedener gewebespezifischer Promotoren (Phloem, Blattmesophyll, Epidermis, Meristem und Wurzel) in *spa*-Mutanten exprimiert und die transgenen Pflanzen auf die Komplementation des Mutanten-Phänotyps hin untersucht. Hierbei konnte gezeigt werden, dass die photoperiodische Regulation der Blütenbildung durch SPA1 ausschließlich im Phloem erfolgt, was darauf hindeutet, dass SPA1 dort zellautonom die Degradation seines Substrates CO, eines positiven Regulators der Blütenbildung, bewirkt. Im Gegensatz dazu beeinflusst SPA1-Aktivität die Blattgröße sowohl im Phloem als auch im Blattmesophyll, jedoch nicht in der Epidermis, was auf nicht-zellautonome Effekte in der SPA1-abhängigen Regulation der Blattgröße hinweist. Darüber hinaus hat die phloem-spezifische Expression von SPA1 einen starken Effekt auf die Keimlingsentwicklung sowohl im Licht als auch in Dunkelheit. Letztlich werden auch die Differenzierung der Spaltöffnungen und die Form der Epidermiszellen über die phloem-spezifische Funktion von SPA1 reguliert. Diese Ergebnisse zeigen, dass Zell-Zell-Kommunikation eine sehr wichtige Rolle in der SPA1-abhängigen Regulation pflanzlicher Entwicklungsprozesse einnimmt.

Die SPA-Proteine, und damit auch der COP1/SPA-Komplex, sind pflanzenspezifisch. Die Funktion des COP1/SPA-Komplexes ist allerdings nur in der zweikeimblättrigen Pflanze Arabidopsis bekannt. Daher wurde in einem zweiten Projekt die Funktion von *COP1*- und *SPA*-Genen des Mooses Physcomitrella und der einkeimblättrigen Pflanze Reis in Arabidopsis getestet. Hierzu wurden zu *COP1* und *SPA* homologe Gene dieser beiden Spezies in Arabidopsis *cop1*- bzw. *spa*-Mutanten exprimiert und die transgenen Pflanzen auf Komplementation des entsprechenden Mutanten-Phänotyps hin untersucht. *COP1*-Homologe aus Reis und Physcomitrella

komplementierten den *cop1*-Mutanten-Phänotyp in Arabidopsis, wohingegen *SPA*-Homologe dieser beiden Spezies die Funktion der Arabidopsis-*SPA*-Gene nicht übernehmen konnten. Dies deutet darauf hin, dass im Falle von COP1 ein grundlegender Signaltransduktions-Mechanismus im Laufe der Evolution konserviert wurde, während die SPA-Proteine unterschiedliche Funktionen übernommen haben. Interessanterweise interagieren COP1- und SPA-Proteine von Physcomitrella *in vitro*, weshalb die Existenz eines COP1/SPA-Komplexes bereits früh in der Evolution der Pflanzen wahrscheinlich ist.

I. Introduction

I. 1. The role of photoreceptors in Arabidopsis development

In their natural habitat, plants are exposed to a variety of environmental factors such as light, temperature and humidity. Being sessile, plants need to adapt to changing environmental factors for their survival. In this direction, plants have evolved an extraordinary degree of developmental plasticity to cope up with the changing environmental conditions. Light is one of the most crucial environmental factors for plant growth and development. It acts not only as a primary source of energy for photosynthesis, but also serves as an informational cue to regulate plant growth and development. Plants monitor light quality, quantity, direction and periodicity to modulate various physiological responses, from seed germination and seedling establishment to mature plant architecture and the onset of reproductive development (Chory et al., 1996; Neff et al., 2000; Casal, 2002). A dramatic effect of light can be observed at the seedling stage, the very early stage of plant development. Seedlings grown in darkness show skotomorphogenesis in displaying an elongated hypocotyl, folded cotyledons, a closed apical hook and no cholorophyll accumulation. In contrast, seedlings grown in the light undergo photomorphogenesis to exhibit a shorter hypocotyl, expanded green cotyledons and an open apical hook (Von Arnim and Deng, 1996; McNellis and Deng, 1995). Seedlings have been used as a model system to elucidate the light signal transduction pathways in plants. Various mutants defective in light signaling, and thus various components of the light signal transduction pathway have been identified using seedling photomorphogenic responses (Reed and Chory, 1994).

Light signal transduction in plants is initiated through photoreceptors perceiving ambient light conditions. Plants have a network of photoreceptors to perceive light quality, quantity and direction (Figure1) (Briggs and Olney, 2001; Sullivan and Deng, 2003; Chen et al, 2004; Franklin et al, 2005; Moeglich et al., 2010). Arabidopsis has four major classes of photoreceptors: phytochromes, cryptochromes, phototropins and the Zeitlupe protein family. The phytochrome family of photoreceptors perceives red (R) and far-red (FR) wavelengths, while the cryptochromes and phototropins detect blue (B) and ultraviolet (UV)-A wavelengths (Franklin and Whitelam, 2003; Lin and Shalitin, 2003; Li and Yang, 2007; Christie, 2007; Franklin and Quail, 2010). The Zeitlupe (ZTL) protein family (ZTL/LKP2/FKF1) may also contribute to blue light perception (Somers et al., 2004; Kim et al., 2007). In plants, UV-B light also triggers developmental responses, however, the UV-B photoreceptor(s) are still unknown (Brown and Jenkins; 2008; Jenkins, 2009).

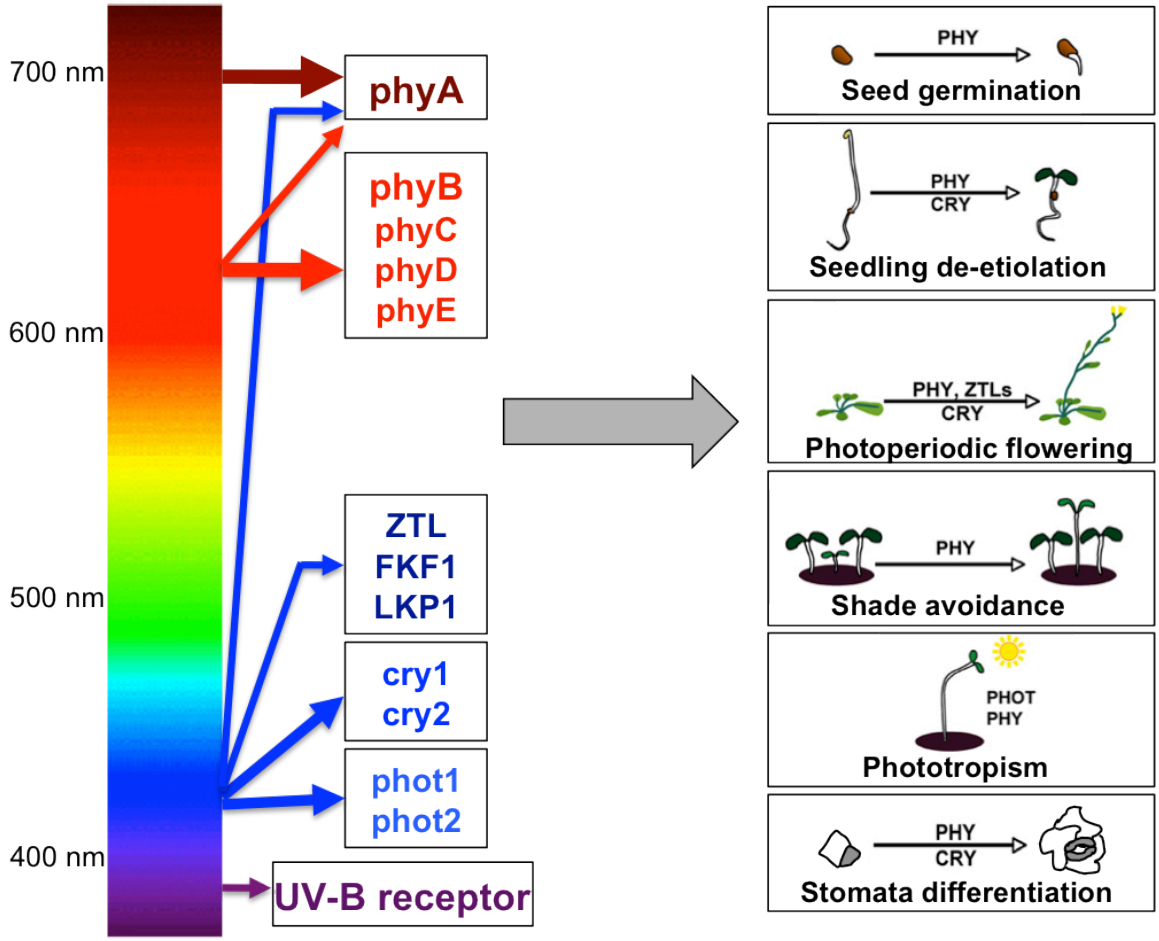

Figure 1: Effects of light on plant growth and development

Different classes of photoreceptors perceive specific wavelengths of light and thereby regulate different stages of plant growth and development. phyA is the sole photoreceptor perceiving continuous FR, besides perceiving B and R. phyB together with phyC, phyD and phyE perceive R. Cryptochromes, phototropins and the Zeitlupe protein family perceive B and UV-A. The UV-B receptor is not known. Seed germination and shade avoidance responses are exclusively regulated by the phytochromes. The phytochromes together with the cryptochromes regulate seedling de-etiolation and stomata differentiation. The phys, crys and ZTLs regulate photoperiodic flowering, whereas the phytochromes together with the phototropins regulate tropic responses.

The phytochromes are by far the most well-studied photoreceptors. They are encoded by a small multigene family in Arabidopsis designated *PHYA* through *PHYE* (Sharrock and Quail, 1989; Clack et al., 1994). phyA is the only photoreceptor to perceive continuous FR in plants, besides mediating the responses to low fluences of R and B (Nagatani et al., 1993; Whitelam et al; 1993). phyB, and to a minor extent phyC, phyD and phyE, mediate the red-light responses in plants (Reed et al., 1993; Quail, 1997). Phytochromes exist as dimers, with each monomer attached to a light-absorbing tetrapyrrole chromophore. They are synthesized in their inactive R-absorbing (Pr) form and biological activity is acquired upon photoconversion to the FR-absorbing (Pfr) form (Kendrick and Kronenberg, 1994). This process of photoconversion also triggers translocation of the phytochromes from the cytoplasm to the nucleus (Sakamoto and

Nagatani, 1996; Nagatani, 2004; Kircher et al., 1999; 2002). In the nucleus, photo-activated phytochromes can directly bind to transcription factors, representing the primary mechanism of phytochrome signaling. Phytochromes can physically interact with bHLH transcription factors, which belong to the family of PHYTOCHROME INTERACTING FACTORS (PIFs) (Ni et al., 1999; Martinez-Garcia et al., 2000; Duek and Fankhauser, 2005). The PIFs mainly act as repressors of light signaling and can be phosphorylated by the phytochromes, which in turn leads to ubiquitination and degradation of the PIFs (Bauer et al., 2004; Al-Sady et al., 2006; Castillon et al., 2007; Shen et al., 2005; 2007; 2008). This basic mechanism enables plants to alter gene expression rapidly in response to light through the phytochromes. The phytochrome – PIF interaction can conversely lead to turnover of the phytochrome photoreceptor, providing a dual mechanism to regulate plant development (Al-Sady et al., 2008).

Arabidopsis has two cryptochromes, cry1 and cry2, with known functions and a third, cry3, with unknown function. cry1 and cry2 are chromoproteins, having two domains: the N-terminal PHR (photolyase homologous region) domain that is responsible for photon absorption, and the C-terminal domain that may act as effector domain. Cryptochromes are mostly nuclear-localized proteins (Lin and Shalitin, 2003; Chen et al., 2004). cry1 shuttles between the nucleus and the cytosol and acts in both places, whereas cry2 is predominantly localized in the nucleus (Cashmore et al., 1999; Wu and Spalding, 2007; Yu et al., 2007). Cryptochromes can also directly interact with transcription factors in the nucleus as photo-activated cry2 interacts with the bHLH transcription factor CIB1 to regulate flowering time (Liu et al., 2008). Different to the cryptochromes, the two phototropins of Arabidopsis, phot1 and phot2, consist of a C-terminal serine/threonine kinase-domain and an N-terminal region containing two LOV sub-domains (Briggs et al, 2001; chen et al, 2004). phot1 and phot2 bind the chromophore flavin mononucleotide through the LOV domains and can undergo B-dependent autophosphorylation. Both phototropins are important for a number of light responses that ultimately allow optimal photosynthesis including phototropism, chloroplast movement and stomatal opening (Liscum and Briggs, 1995; Briggs and Christie, 2002; Ohgishi et al., 2004). The ZTL photoreceptors also have an N-terminal LOV domain similar to phototropins and these photoreceptors regulate light input to the circadian clock and the photoperiodic flowering pathway (Schultz et al., 2001; Imaizumi et al., 2003; Somers et al., 2004).

The roles of photoreceptors in mediating plant development have been confounded by the crosstalk in photoreceptor signaling. The photoreceptors show redundant, synergistic and sometimes mutually antagonistic mechanisms of action (Figure 1) (Sullivan and Deng, 2003; Chen et al, 2004; Franklin et al, 2005). With the

exception of seed germination and shade avoidance response, which are controlled solely by phytochromes in Arabidopsis, other physiological processes including seedling development and floral induction, are controlled by interconnected networks of both phytochromes and cryptochromes (Ahmad and Cashmore, 1997; Devlin and Kay, 2000; Casal, 2000; Mazella et al, 2001; Franklin et al., 2005). phyA and phyB are the major photoreceptors, with a significant contribution from phyE, to induce seed germination in Arabidopsis (Casal and Sanchez, 1998; Henning, 2002). phyB has a predominant role in regulating germination in red light, whilst phyA induces germination in low fluences of red or far-red light or continuous far-red light. Following seed germination, light signaling results in the inhibition of hypocotyl elongation and the expansion of cotyledons for normal seedling establishment, a process termed as de-etiolation. Both phytochromes and cryptochromes are implicated in seedling de-etiolation by sensing specific light quality and quantity. Both cry1 and cry2 regulate seedling de-etiolation, but cry1 has a major function under high fluences of B and cry2 is the prime regulator under low fluences of B (Ahmad and Cashmore, 1993; Ahmad et al., 1995, Lin et al., 1996, Lin et al., 1998). Among the phytochromes, phyA and phyB are the most important to regulate seedling de-etiolation. phyA primarily mediates seedling de-etiolation in response to FRc (Nagatani et al., 1993; Whitelam et al; 1993) and, besides, phyA also mediates responses to low fluences of red and blue light. In contrast, phyB regulates seedling de-etiolation in response to red light (Nagatani et al., 1991; Reed et al., 1993). Thus, the light-mediated seedling de-etiolation involves a complex interplay of both phytochromes and cryptochromes. Consistent with this, a physical interaction between cry1 and phyA proteins has been demonstrated in addition to a functional interaction between cry2 and phyB (Ahmad et al., 1998; Mas et al., 2000). Eventually, the combined action of phyA, phyB, cry1 and cry2 regulates the stimulation of cholorophyll synthesis by light (Reed et al., 1994; McCormac and Terry, 2002).

The effect of photoreceptors in regulation of adult plant and leaf development is best characterized in the shade avoidance syndrome. This syndrome results from a reduced R:FR radiation through shading by neighboring vegetation and is characterized by stimulated elongation growth, reduced leaf development, increased apical dominance and reduced branching (Franklin, 2008). Phytochromes are known to be exclusively responsible for this response. (Devlin et al., 1996; Whitelam and Devlin, 1997). phyB plays a predominant role in this process since Arabidopsis *phyB* mutant plants show constitutive shade avoidance responses, such as elongated stems and reduced leaf size (Nagatani et al., 1991). In addition, phyD and phyE also contribute to these responses (Franklin et al., 2003). Enrichment of FR in transmitted/reflected light can lead to the action of phyA in high irradiance mode to inhibit the elongation growth, thereby limiting

shade avoidance responses (Johnson et al., 1994; Salter et al., 2003). Further, phyA, phyB and phyE function redundantly to maintain the compact rosette habit of Arabidopsis (Devlin et al., 1998). Additionally, stomata development in Arabidopsis requires the presence of light and is dependent on the presence of photoreceptors. Cryptochromes mediate blue light-induced stomata development, whereas phyA and phyB mediate red and far-red light-induced stomata development, respectively, and all these photoreceptors act together to promote stomata development (Kang et al., 2010). Further, phototropins also regulate blue light-induced opening of stomata (Kinoshita et al., 2001). The molecular mechanism of photoreceptor-regulated stomata development is unknown.

Photoreceptors have a great impact on the regulation of photoperiodic flowering in Arabidopsis (Guo et al., 1998; Mockler et al., 2003; Valverde et al., 2004; Imaizumi and Kay, 2006). Arabidopsis is a facultative long day plant, which flowers earlier in long days but eventually flowers even under short days (Coupland et al., 1998). CONSTANS (CO) is the key transcription factor inducing flowering specifically under long days in Arabidopsis (Putterill et al., 1995). CO activates transcription of *FLOWERING LOCUS T (FT)* in leaf companion cells, and then the FT protein moves through the phloem to induce flowering at the shoot apex (Corbesier et al., 2007; Turck et al., 2008). At the shoot-apex, FT interacts with the transcription factor FLOWERING LOCUS D (FD) to regulate expression of floral genes (Samach et al., 2000; Abe et al., 2005; Yoo et al., 2005). *CO* transcription is regulated by the circadian clock, and the external coincidence model suggests that flowering is induced in long days because CO expression coincides with the exposure of plants to light (Searle and Coupland, 2004; Imaizumi and Kay, 2006). Further, light-mediated post-transcriptional regulation of CO is essential for photoperiodic flowering (Valverde et al., 2004). phyA, phyB and cry2 regulate photoperiodic flowering by regulating CO stability. phyA and cry2 stabilize CO in the presence of light, whereas phyB promotes degradation of CO (Valverde et al., 2004). Hence, phyA and cry2 promote flowering in long days, whilst phyB inhibits flowering (Guo et al., 1998; Mockler et al., 1999; Mockler et al., 2003).

Thus, the multiplicity of responses to environmental light signals available to plants results from the combined action of all photoreceptors. Redundancy, synergism and antagonism among them increase the sensitivity of plants to changing light conditions and permit an array of developmental responses.

I. 2. The COP1/SPA complex: Repressor of light signaling in darkness

Regulated protein degradation plays a central role in the light signal transduction pathway. Light regulates photomorphogenesis by inhibiting ubiquitination and subsequent degradation of the transcription factors required for light signaling (Hoecker, 2005; Henriques et al., 2009). Downstream of photoreceptors, a group of 11 *CONSTITUTIVELY PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP/DET/FUS)* genes are necessary for repression of photomorphogenesis in darkness (Chory et al., 1989; Wei et al., 1996; Kim et al., 2002). These genes encode negative regulators of light control of plant development. Seedlings with mutations in any of these genes show constitutive photomorphogenesis in darkness in displaying short hypocotyls and open cotyledons. These mutants have strongly elevated levels of key photomorphogenesis-promoting transcription factors such as HY5 and HFR1 in darkness (Osterlund et al., 2000a; Osterlund et al., 2000b; Duek et al., 2004; Yang et al., 2005).

Among the *COP/DET/FUS* genes, *COP1* is the most well characterized. It encodes a light-inactivatible repressor of photomorphogenesis, which contains a WD-repeat domain, a coiled-coil domain, and a RING finger typical of a subclass of E3 ubiquitin ligases (Figure 2A) (Deng et al., 1991, Deng et al., 1992, Osterlund et al, 1999; Yi and Deng, 2005). Indeed, COP1 has E3 ubiquitin ligase activity. It suppresses photomorphogenesis in darkness by promoting the ubiquitination followed by subsequent degradation of the activators of the light response, such as HFR1, HY5, HYH and LAF1 (Osterlund et al., 2000a; Seo et al., 2003; Jang et al., 2005; Yang et al.; 2005). The *cop1* mutant, therefore, undergoes constitutive photomorphogenesis in darkness and displays the features of light-grown seedlings under these conditions (Figure 3B) (Deng, 1991). Consistent with this, the genome expression profile of the *cop1* mutant in darkness mimics that of light-grown seedlings (Ma et al., 2002). In the light, photo-activated photoreceptors inhibit the COP1 function through a yet unknown mechanism, and hence the transcription factors required for light signaling are stabilized and normal photomorphogenesis takes place. One of the mechanisms of light-mediated inhibition of COP1 function is by changing the subcellular localization of COP1. COP1 is primarily present and functional in the nucleus in darkness. However, light mediates the exclusion of COP1 from the nucleus in a process that is initiated by photoreceptor signaling (Von Arnim and Deng, 1994; Von Arnim et al., 1997; Subramaniam et al., 2004). Moreover, phytochromes and cryptochromes have been shown to physically interact with COP1, and thus light-dependent inactivation of COP1 might also result from its physical interaction with photoreceptors (Wang et al., 2001; Yang et al., 2001; Seo et al., 2004). In turn, phyA undergoes COP1-dependent ubiquitin-mediated degradation in the light, which desensitizes phyA-mediated signaling (Seo et al., 2004). Further, blue light-mediated

degradation of cry2 also requires COP1 (Shalitin et al., 2002). Recently, COP1 has also been shown to act as an E3 ubiquitin ligase for phyB and targets nuclear phyB for degradation (Jang et al., 2010). Similarly phyC, phyD and phyE are also targeted by COP1. In contrast to the degradation of photomorphogenesis-promoting transcription factors in darkness, seedling skotomorphogenesis is also mediated by stabilization of PIF3 in darkness. Unexpectedly, COP1 promotes PIF3 stabilization in darkness through an unknown mechanism (Bauer et al., 2004; Park et al., 2004). PIF3 degradation in the light is independent of COP1, suggesting the involvement of another ubiquitin ligase in this process.

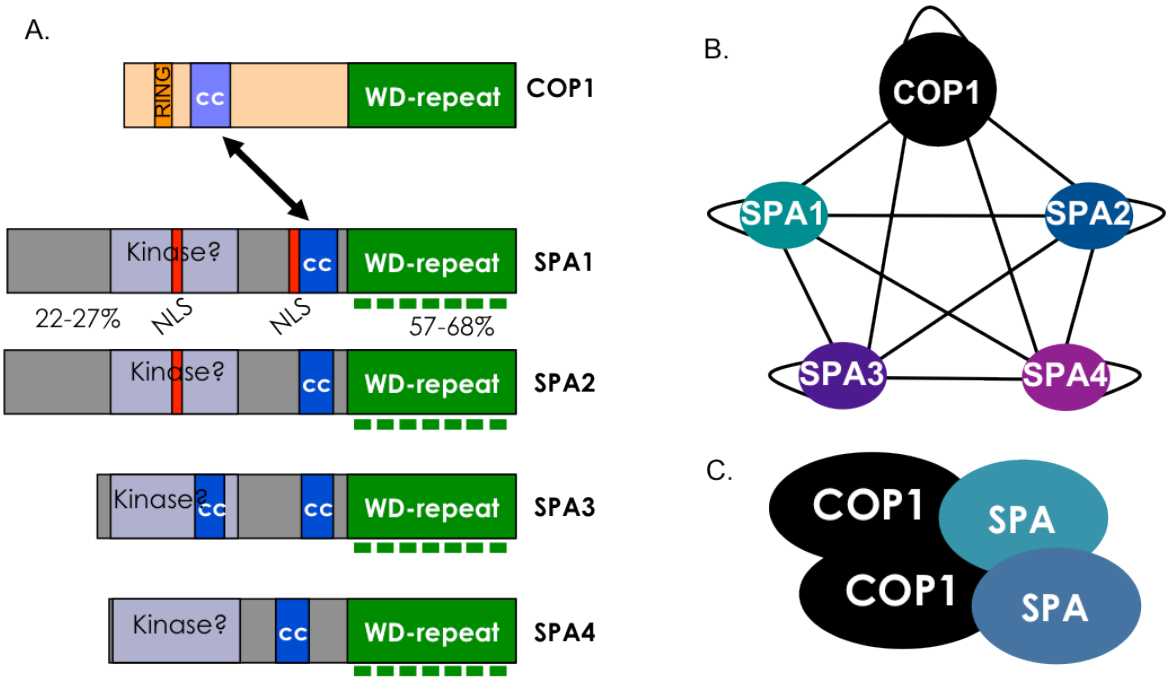

Figure 2: SPA proteins interact with COP1 to form multiple COP1/SPA complexes in Arabidopsis.

(A) The COP1 protein consists of a RING-finger, a coiled-coil (CC) and a WD-repeat domain. All SPA proteins have a kinase-like domain, a coiled-coil and a WD-repeat domain. SPA1 and SPA2 also have nuclear localization sequences (NLS). SPA proteins show 57-68% similarity in their WD-repeat domain and 22-27% similarity at the amino terminus. All SPA proteins can interact with COP1 via their respective coiled-coil domains.
(B) All SPA proteins can self-associate in vivo to form homodimers or can interact with other SPA proteins and the COP1 protein to form heterodimers. COP1 can also self-associate to form homodimers.
(C) The postulated COP1/SPA complexes are tetramers with a COP1 homodimer and a SPA homo/heterodimer.

The COP1 E3 ubiquitin ligase requires the presence of SUPPRESSOR OF PHYTOCHROME-A (SPA) proteins to promote degradation of photomorphogenesis-promoting transcription factors in darkness (Hoecker and Quail, 2001; Hoecker, 2005; Lin and Wang, 2007; Zhu et al., 2008). All four members of the SPA protein-family (SPA1-SPA4) are essential for repression of photomorphogenesis in darkness. In darkness, the

spa1 spa2 spa3 spa4 quadruple mutant displays strong constitutive photomorphogenesis and elevated levels of transcription factors required for light signaling, similar to the *cop1* mutant (Laubinger et al., 2004). The first member of the *SPA* gene-family, *SPA1*, was initially identified as a suppressor of a weak *phyA* mutation. The *spa1* mutant shows exaggerated photomorphogenesis in FR, R and B, but is indistinguishable from the wild type in darkness (Hoecker et al., 1998). *spa1* mutants require the presence of a functional *PHYA* gene to show their mutant phenotype, suggesting that SPA1 is important for normal phyA signaling. Further, SPA1 has been suggested to counteract the phytochrome-mediated inhibition of hypocotyl elongation (Parks et al., 2001). *SPA1* encodes a nuclear localized protein having an N-terminal kinase-like domain, a central coiled-coil domain and a C-terminal WD-repeat domain (Figure 2A) (Hoecker et al., 1999). The WD-repeat domain of SPA1 shows close similarity with the COP1 WD-repeat domain. Genetic interaction between *spa1* and *cop1* mutations and direct physical interaction between SPA1 and COP1 via their respective coiled-coil domains suggest that these proteins act together to suppress photomorphogenesis in darkness (Saijo et al., 2003). Indeed, SPA1 modulates the E3 ubiquitin ligase activity of COP1. Moreover, SPA1 physically interacts with targets of COP1 activity (HY5 and HFR1) via the WD-repeat domain and controls the stability of these transcription factors (Saijo et al, 2003; Seo et al; 2003, Jang et al; 2005).

Arabidopsis has three SPA1-like proteins: SPA2, SPA3 and SPA4 (Laubinger and Hoecker, 2003; Hoecker, 2005). All four SPA proteins contain a similar domain structure with a kinase-like motif, a coiled-coil domain and a WD-40 motif (Figure 2A). SPA proteins are highly similar within their WD-repeat domains (57-68% identical amino acids), whereas less sequence similarity is observed in the N-terminus (22-27% identical amino acids) (Laubinger and Hoecker, 2003). The *SPA* gene family can be subdivided into two subclasses based upon their sequence similarity, *SPA1* and *SPA2* being in one subclass and *SPA3* and *SPA4* in the other (Hoecker, 2005). SPA1 and SPA2 show highest sequence similarity with similar protein size and fully conserved locations of splice sites within the coding region. SPA3 and SPA4 proteins are highly conserved having 74% identical amino acids.

All four SPA proteins have been shown to interact with COP1 via their coiled-coil domain (Figure 2B). SPA proteins are critical for COP1 activity in vivo. Furthermore, SPA proteins can also self-associate both in vivo and in vitro (Laubinger and Hoecker, 2003; Laubinger et al., 2004; Zhu et al., 2008). COP1 and the four SPA proteins are suggested to form a group of heterogeneous COP1/SPA complexes in Arabidopsis. The COP1/SPA complex is postulated to have a COP1 homodimer and a SPA homo/heterodimer (Figure 2C) (Zhu et al, 2008). The exact composition of COP1/SPA complexes might well depend

upon the light conditions, developmental stages and tissue types. Together the COP1/SPA complex constitutes the functional E3-ubiquitin ligase, and thus it targets the photomorphogenesis-promoting transcription factors for degradation in darkness (Figure 3A).

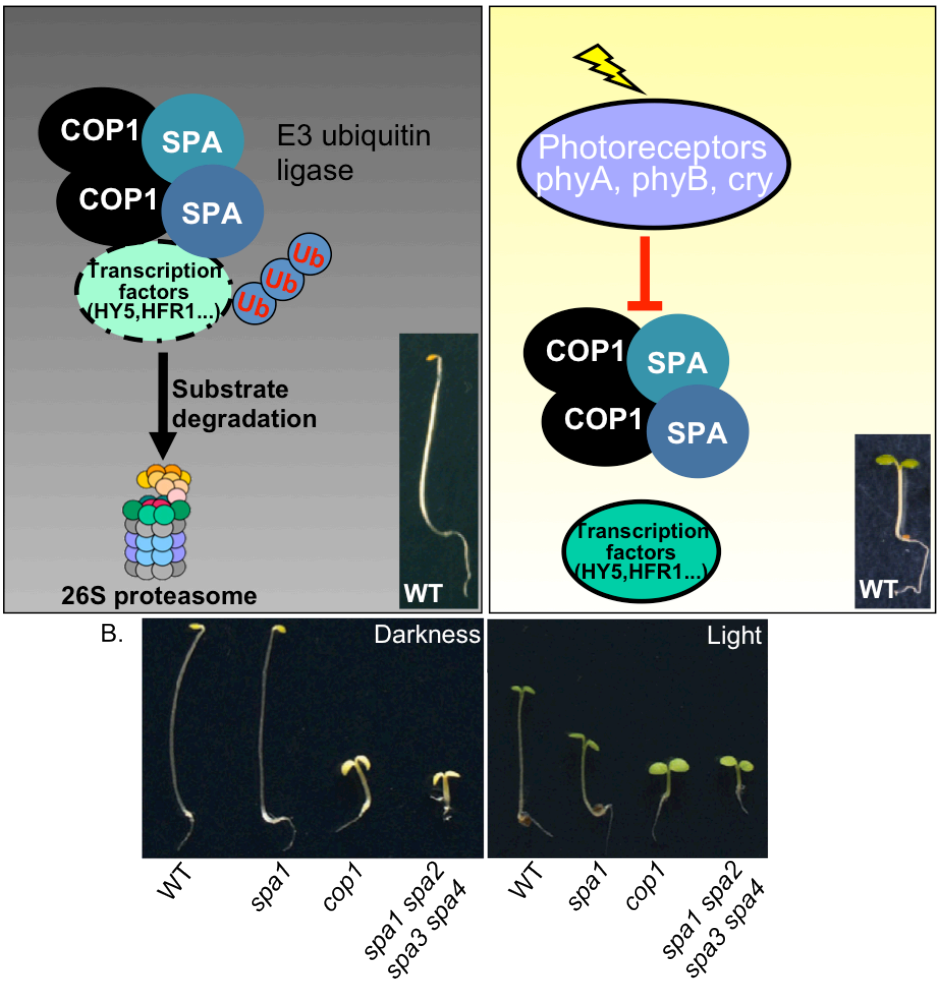

Figure 3: The COP1/SPA complex functions as a repressor of light signaling.

(A) The COP1/SPA complex functions as a ubiquitin ligase. It promotes the ubiquitination of photomorphogenesis-promoting transcription factors in darkness. The ubiquitinated transcription factors are subsequently degraded via the 26S proteasome, which leads to skotomorphogenesis in dark-grown seedlings. In light, activated photoreceptors inhibit the COP1/SPA function through an unknown mechanism, and hence the transcription factors are stabilized, leading to photomorphogenesis.
(B) Visual phenotype of wild-type (WT), *spa1*, *cop1*, *spa1 spa2 spa3 spa4* mutant seedlings in darkness and under red light. Dark-grown wild-type seedlings show elongated hypocotyls and closed cotyledons, whereas light-grown wild-type seedlings display reduced hypocotyl length and expanded green cotyledons. The *spa1* mutant does not show any phenotypic defect in darkness, but shows exaggerated photomorphogenesis in light. *cop1* and *spa1 spa2 spa3 spa4* mutant seedlings show constitutive photomorphogenesis in light and darkness (photographs taken from Hoecker, 2005).

I. 3. *SPA* genes regulate various stages of plant growth and development

Comparative analysis of *spa* double and triple mutants uncovered redundant as well as specific functions of the four *SPA* genes in the regulation of plant growth and development. *spa* single mutant seedlings do not show any obvious phenotypic defect in darkness and they appear similar to wild-type seedlings (Hoecker et al., 1998; Laubinger and Hoecker, 2003). Increasing the *spa* mutation levels to double and triple mutants tends to increase the features of light-grown seedlings in darkness, with the *spa1 spa2 spa3 spa4* quadruple mutant showing an extreme constitutive photomorphogenic phenotype (Figure 3B, 4) (Laubinger et al., 2004). This indicates functional redundancy among the *SPA* genes to regulate seedling skotomorphogenesis. Analysis of *spa* triple mutants, with only one functional *SPA* gene, further indicates that *SPA1* and *SPA2* are the predominant regulators of seedling development in darkness. *SPA* genes also regulate seedling development in light (Figure 3B). *spa1*, *spa3* and *spa4* single mutants show exaggerated photomorphogenesis in R, FR and B , with the *spa2* mutant showing no visible defect (Laubinger et al., 2004; Fittinghoff et al., 2006). Further analysis of *spa* triple mutants revealed that *SPA1* is the prime regulator of seedling development in light with contributions from *SPA3* and *SPA4*. *SPA2* has little function in light-grown seedlings (Laubinger et al., 2004).

Adult *spa* quadruple mutant plants are extremely dwarfed with tiny leaves, similar to the *cop1* mutant (Figure 4). *spa3 spa4* double mutants also show reduced plant size, whereas triple mutants having either functional *SPA3* or *SPA4* show plant sizes almost similar to wild-type plants. This indicates that *SPA3* and *SPA4* are the major regulators of adult plant and leaf size development (Laubinger et al., 2004; Fittinghoff et al., 2006). *SPA1* has a significant contribution in this developmental process, while *SPA2* has very little function in adult plants. Substrates of the SPA proteins involved in the regulation of adult plant and leaf size are thus far unknown.

Recently, *COP1* and the *SPA* genes have also been shown to be involved in stomata development in Arabidopsis. Wild-type seedlings fail to fully differentiate stomata in darkness, while the *cop1* and the *spa1 spa2 spa3* triple mutant show constitutive stomata development in darkness (Figure 4) (Kang et al., 2009). This indicates that the COP1/SPA complex acts as a repressor of stomata development in darkness.

SPA genes are essential for photoperiodic flowering in Arabidopsis as they repress flowering in non-inductive short days (Figure 4) (Ishikawa et al., 2006; Laubinger et al., 2006). The *spa1* mutant flowers earlier than the wild type in short days, indicating that *SPA1* is the predominant regulator of photoperiodic flowering. The *spa2 spa3 spa4* triple mutant, having only functional *SPA1*, flowers like the wild type, indicating that *SPA1* alone is sufficient to repress photoperiodic flowering. Further, the *spa1 spa3 spa4* triple

mutant, having only functional *SPA2*, shows extremely early flowering as it flowers almost at the same time in both long and short days, demonstrating that *SPA3* and *SPA4* may also contribute to photoperiodic flowering, while *SPA2* has no or a very minor role in this process (Laubinger et al., 2006). SPA-regulated photoperiodic flowering operates through the regulation of CO stability. SPA1 as well as COP1 physically interact with CO. *spa1* and *spa1 spa3 spa4* mutants show increased CO stability, which in turn is reflected in terms of increased *FT* transcript levels leading to the early-flowering phenotype of these mutants (Ishikawa et al., 2006; Laubinger et al., 2006; Jang et al., 2008). Genetic studies showed that the early-flowering phenotype of the *spa1* mutant is dependent upon the presence of functional *CO* (Laubinger et al., 2006). Taken together, these observations suggest that the COP1/SPA complex regulates photoperiodic flowering by regulating CO stability through the ubiquitin – proteasome pathway.

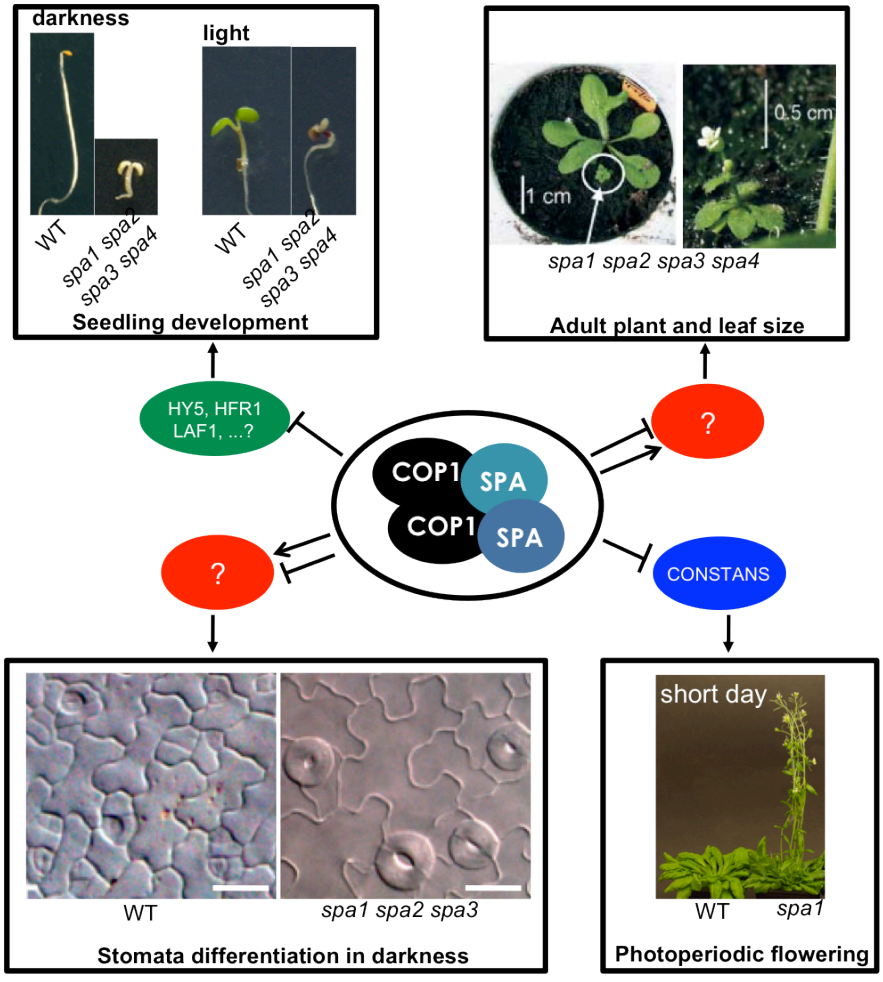

Figure 4: SPA-regulated plant developmental processes in Arabidopsis.

SPA proteins regulate seedling development, stomata differentiation, adult plant size and photoperiodic flowering in Arabidopsis. The COP1/SPA complex targets transcription factors such as HY5 and HFR1 for repressing seedling photomorphogenesis and CO for repressing flowering in short days. Substrates for the regulation of leaf size and stomata differentiation are unknown. (photographs taken from Laubinger et al., 2004; Laubinger et al., 2006 and Kang et al., 2009).

Altogether, SPA proteins regulate various stages of plant growth and development with redundant as well as specific functions of the individual *SPA* gene-family members. In spite of the observed redundancy and specificity, SPA1 plays an important role in all SPA-regulated plant developmental processes. SPA1 is primarily important for seedling development in darkness and light as well as for photoperiodic flowering. In addition, it also has a significant role in adult plant size determination. SPA2 has a major function in dark-grown seedlings, while SPA3 and SPA4 are primarily responsible for regulating adult plant and leaf size.

I. 4. Cell-cell communication in light-regulated plant development

Light may affect gene expression and plant developmental processes away from the site of perception through intercellular and/or interorgan communication. Plant leaves are highly optimized to sense and absorb the maximum of light, but the light responses must be manifested throughout the plant. Consistent with this, the sites of light perception and light action do not always overlap in plants, highlighting the importance of long-distance communication in light signaling (Bou-Torrent et al., 2008; Montgomery et al., 2008). Intercellular communication in light-regulated gene expression has been elegantly demonstrated through localized micro-beam irradiation of small areas within the tobacco cotyledon. When only a few cells were irradiated, the expression of photosynthetic genes was induced not only in the irradiated cells, but also in cells not receiving the light stimulus (Figure 6A) (Bischoff et al., 1997). Similarly, light treatments of cotyledons can induce light-responsive transgene expression in the hypocotyls of transgenic Arabidopsis (Tanaka et al., 2002).

Photoperiodic induction of flowering is the best-studied example of non-cell autonomous effects in light-regulated plant developmental processes (Figure 5) (Turck et al., 2008). Classical physiological experiments suggest the movement of one or more systemic signals from leaves through the phloem to induce flowering at the shoot apex (Zeevaart, 1976). Recent genetic and molecular studies nicely establish the long distance signaling involved in photoperiodic flowering in Arabidopsis. Leaves perceive the day-length, and then a mobile signal, the FT protein, moves through the phloem to induce flowering at the shoot apex (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007). Consistent with this, CO, the transcription factor regulating *FT* transcription, also acts in the phloem to regulate photoperiodic flowering (An et al., 2004). Upstream of CO, the photoreceptor cry2 also acts in the phloem to promote flowering in long days (Endo et al., 2007). In contrast, phyB acts in mesophyll cells to inhibit this process (Endo et al., 2005). Thus, non-cell autonomous effects from both the phloem and the mesophyll operate together to regulate photoperiodic flowering.

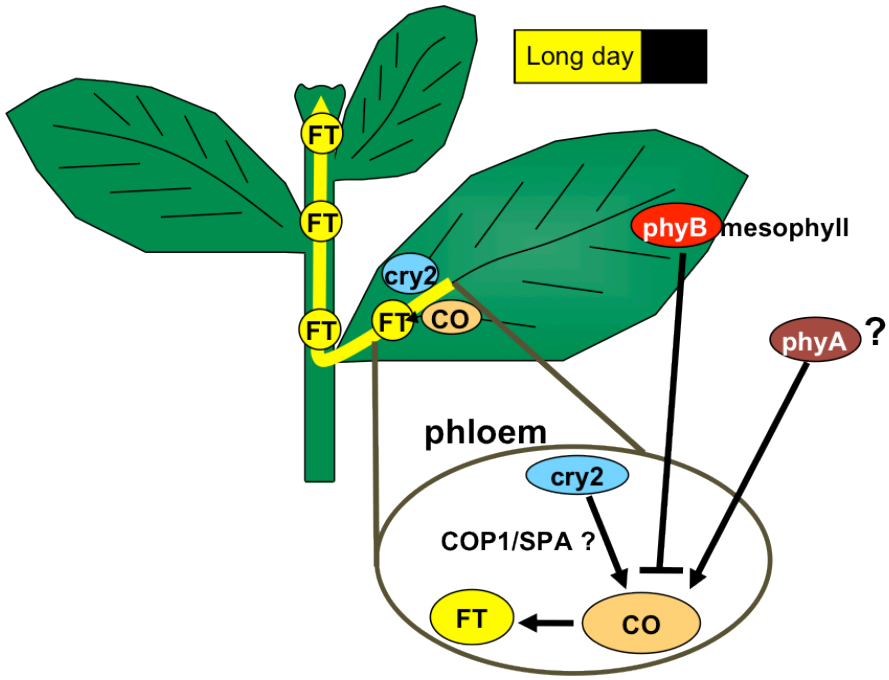

Figure 5: Cell-cell communication in photoperiodic flowering of Arabidopsis.

CO acts in the phloem to induce *FT* transcription, and then the FT protein moves through the phloem to induce flowering at the shoot apex. cry2 also acts in the phloem to regulate this process. phyB, in contrast, acts in the mesophyll to promote CO degradation in the phloem. The functional site of phyA to regulate flowering is not known.

The long distance signaling is also employed in photoreceptor-mediated inhibition of hypocotyl length. Red light strongly inhibits the hypocotyl elongation in cucumber and cotyledon-localized phyB has been implicated in this process (Black and Shuttleworth, 1974). In Arabidopsis, expression of a phyB-GFP fusion in cotyledons can complement the long hypocotyl phenotype of the *phyB* mutant under white light (Figure 6B) (Endo et al., 2005). Recently, red and far-red light perception by cotyledon-localized phytochromes has been shown to be essential for the inhibition of hypocotyl elongation in Arabidopsis. Inhibiting the phytochrome function specifically in the cotyledons results in elongation of the hypocotyl in the light (Warnasooriya et al., 2009). In both previous studies, phytochrome activity in mesophyll cells of cotyledons has been shown to regulate hypocotyl elongation. Taken together, these observations suggest the involvement of cotyledon-localized phytochrome-dependent signals in the inhibition of hypocotyl elongation in response to light. Also in adult mustard plants, the stem elongation response, a characteristic of the shade avoidance syndrome, is mediated through light perception by leaf-localized phytochromes (Casal and Smith, 1988). Similarly in Arabidopsis, cotyledons/leaves perceive the reduced R:FR ratio that induces the elongation response of stem and petiole, characteristic for the shade avoidance syndrome (Tao et al., 2008). Besides the cell-cell communication for the elongation

response, photoactivation of phytochromes in the cotyledons has been observed to regulate light-dependent apical hook opening (Powell and Morgan, 1980). Additionally, phytochrome activity in both leaves and the stem is required for proper leaf expansion in adult plants (De Greef and Caubergs, 1972).

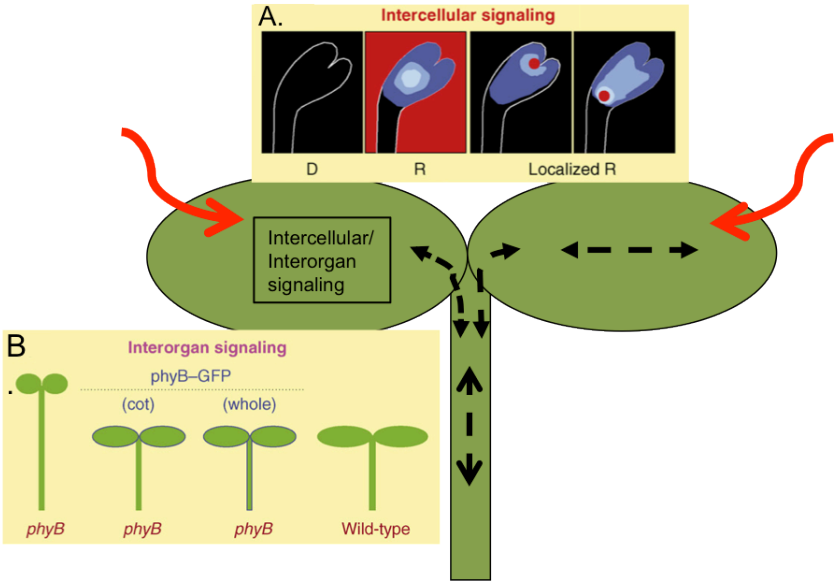

Figure 6: Intercellular and interorgan communications in light responses of seedlings. (Picture modified from Bau-Torrent et al., 2009)

(A) Intercellular signaling: A localized red light (R) irradiation on the cotyledons of dark-grown seedlings induces the expression of light-induced genes in cells not receiving the light stimulus. The red dot indicates the point of localized R irradiation and the blue coloring indicates the expression of light-induced genes (light blue = highest, dark blue = lowest). D represents darkness.
(B) Interorgan signaling: Expression of a phyB-GFP fusion complements the long-hypocotyl phenotype of the *phyB* mutant both when expressed only in the cotyledons (cot) or in the whole seedling. Arrows indicate unidentified long-distance signaling molecules moving between cotyledons and the hypocotyl to regulate hypocotyl elongation.

The exact nature of these long distance signals in light-regulated leaf and seedling development is largely unknown. However, there is evidence for the involvement of phytohormones, especially auxin, in light-mediated elongation responses i.e. hypocotyl elongation at the seedling stage and petiole/stem elongation at the adult plant stage. Cross talk between light and phytohormone signaling in the regulation of plant development is well known by now. Auxin has been implicated as one of the mediators of phytochrome-dependent processes (Halliday et al., 2009). IAA proteins, which are the major auxin signaling components, have been reported to physically interact with phytochromes (Colon-Carmona et al., 2000; Tian et al., 2003). Consistent with this, various *iaa* gain-of-function mutants like *axr2*, *axr3* and *shy2* show constitutive photomorphogenesis in darkness (Leyser et al., 1996; Reed et al., 1998; Nagpal et al., 2000). Due to its characteristic polar transport, auxin can move from cell to cell in a

directed fashion, and it may constitute an important component of the non-cell autonomous signals generated by light. Indeed, auxin transport in the hypocotyl has been shown to be phytochrome dependent (Jensen et al., 1998; Mazzella et al., 2000). In the same direction, shoot-localized phytochromes regulate auxin transport from shoot to root, thereby effecting lateral root development and auxin-induced gene expression in roots (Salisbury et al., 2007). Auxin has further been implicated in the induction of light-responsive transgene expression in the hypocotyl through cotyledon-localized phytochromes (Tanaka et al., 2002).

Recently, the shade-avoidance syndrome signal, the low R:FR ratio, has been shown to induce auxin biosynthesis through a new pathway primarily in cotyledons and then transport of free auxin from the cotyledons to the hypocotyl leads to elongation growth (Tao et al., 2008). Also, auxin, together with another critical hormone, cytokinin, is involved in this arrest of leaf growth which is associated with the shade avoidance syndrome. The leaf growth arrest is caused by localized auxin-induced cytokinin breakdown in incipient vein cells of developing primordia (Carabelli et al., 2007). Besides auxin and cytokinin, brassinosteroids are also involved in the non-cell autonomous regulation of hypocotyl length as well as adult plant size. Brassinosteroids act in the epidermis to regulate leaf size as well as hypocotyl length (Savaldi-Goldstein et al., 2007). Altogether these observations suggest a close link between light and phytohormone signaling and hormones may constitute an important component of the non-cell autonomous effects in light signaling.

I. 5. Light signaling in rice and Physcomitrella

Light signaling through the involvement of photoreceptors and downstream signaling components has been extensively studied in Arabidopsis, but not much is known about light signaling in other plant species (Chen et al., 2004). However, the light signal transduction mechanism appears to be, at least in part, conserved during evolution as all plant species right from unicellular algae to moss and flowering angiosperms possess light-sensing photoreceptors (Lariguet et al., 2005; Bae et al., 2008; Moeglich et al., 2010). Interestingly, the Chlamydomonas photoreceptor phototropin as well as the CONSTANS homolog are functional in Arabidopsis, suggesting a similar basic mechanism of action of these light signaling components in plants during evolution (Onodera et al., 2005; Serrano et al., 2009).

Similar to Arabidopsis, which is a dicotyledonous plant, light immensely affects the developmental processes in monocotyledonous plants such as rice. There, light regulates seed germination, elongation of coleoptiles and mesocotyls, unrolling of the leaf blade, plastid development and flowering (Kendrick and Kronenberg, 1994; Biswas et al., 2003;

Smith et al., 2006). Although many classical physiological studies of photomorphogenesis have been done using monocots, light signal transduction and its components are not very well understood in these plants. The mechanisms of photoreceptor function and photoperiodic flowering in rice share similarity to those in Arabidopsis. Photoperiodic flowering in rice has been studied in more detail and involves components of photoperiodic flowering that are conserved between the two species, in spite of rice being a short-day plant (Izawa et al., 2003; Hayama and Coupland, 2004). In rice *Hd1* encodes the *CO* homolog and *Hd3a* encodes an *FT* homolog. However, the role of Hd1 in rice is more complex than that of CO in Arabidopsis as Hd1 promotes *Hd3a* expression and flowering under short days and inhibits *Hd3a* expression and flowering under long days (Yano et al., 2000; Izawa et al., 2002; Kojima et al., 2002). Furthermore, rice also has a similar set of photoreceptors. Rice has three phytochromes: phyA, phyB and phyC, which are the sole photoreceptors for perceiving R/FR (Takano et al., 2005 and 2009). The rice phytochrome triple mutant shows skotomorphogenesis under continuous red or far-red light, elongated internodes during the vegetative growth stage and also an early-flowering phenotype under long days. The latter is consistent with the suggestion of the phytochromes being involved in Hd1-mediated inhibition of *Hd3a* expression, and thus in flowering in long days (Izawa et al., 2000 and 2002). The three cryptochromes of rice, cry1a, cry1b and cry2, are functionally similar to Arabidopsis cryptochromes. Both cry1 are responsible for blue light-mediated de-etiolation in rice, whereas cry2 is involved in the promotion of flowering (Hirose et al., 2006; Zhang et al., 2006). Rice also has two phototropins, phot1 and phot2, although the functional significance of these phototropins in rice is not known in detail (Kasahara et al., 2002).

Recently the moss species *Physcomitrella patens*, because of the possibility of generating targeted knockouts, has emerged as a model organism to study the signaling pathways in plants early in evolution (Cove et al., 2006). Specific chromosomal genes can be disrupted via homologous recombination in Physcomitrella, thereby facilitating the study of individual gene functions. Moreover, protonemal filaments are gametophytic and thus haploid, allowing the results of genetic lesions to be observed immediately. While mosses are not able to etiolate, they certainly show light responses and harbor a full complement of photoreceptors. Light-mediated responses in Physcomitrella include induction of branching on the protonema, phototropism of protonemal filaments, gametophore induction and development, and chloroplast relocation (Wada and Kadota, 1989; Kasahara et al., 2004; Mittmann et al., 2004; Uenaka and Kadota, 2007). Physcomitrella has four phytochromes: phy1 – phy4. They are primarily involved in protonema phototropism and chloroplast relocation in response to polarized red light. Interestingly phytochrome localization in Physcomitrella is not changed by light and

phytochromes are functional in the cytoplasm (Mittmann et al., 2004; Uenaka et al., 2005; Uenaka and Kadota, 2007). Besides, Physcomitrella has two almost identical cryptochromes: cry1a and cry1b, which are primarily involved in the induction of branches on the protonema and in the induction and development of gametophore (Imaizumi et al., 2002). Interestingly, Physcomitrella *cry* mutants are hypersensitive to auxin in a blue-light specific way, suggesting a cross talk of light and phytohormone signaling even early in evolution (Imaizumi et al., 2002). Finally, the four Physcomitrella phototropins (photA1, photA2, photB1 and photB2) have roles in chloroplast movement as well as in branching of the protonema (Kasahara et al., 2004).

In spite of characterized functions of photoreceptors in rice and Physcomitrella, signal transduction downstream of these photoreceptors is largely unknown. COP1 and the SPA proteins are important components of light signaling downstream of photoreceptors in Arabidopsis (Hoecker, 2005). COP1 is more diverse in evolution as it is present even in mammals (Yi and Deng, 2005). Mammalian COP1 fails to complement the Arabidopsis *cop1* mutant phenotype. However, mammalian COP1 changes sub-cellular localization in response to light in Arabidopsis, suggesting that mammalian COP1 may share a common mode of action with its Arabidopsis counterpart (Wang et al., 1999). In contrast, SPA proteins are plant-specific. Therefore, the COP1/SPA complex is also a plant-specific entity. Previous study demonstrated the presence of one *COP1* homolog in rice, which is functional in Arabidopsis seedlings (Tsuge et al., 2001). Further rice cry1 has been suggested to involve direct interaction with COP1 for its function, as in Arabidopsis (Zhang et al., 2006). In contrast, there is no information about *SPA* genes in rice. In *Physcomitrella patens*, phylogenetic studies suggest the presence of one *SPA* and nine *COP1* homologs (Richardt et al., 2007). The functional significance of these *COP1* and *SPA* genes in Physcomitrella and their integration with photoreceptor signaling is completely unknown.

II. Aims of this PhD thesis

i) Cell-cell communication in SPA1-regulated plant development: It is now well documented that light initiates developmental responses through non-cell autonomous signaling. I, therefore, wished to investigate whether the light signaling intermediate SPA1 participates in or induces non-cell autonomous signaling events. Therefore, the primary aim of this thesis was to investigate the functional sites of SPA1 to regulate different plant developmental processes. To this end, a β-glucuronidase(GUS)-SPA1 fusion protein was expressed under the control of different tissue-specifically expressed promoters in *spa* mutant plants. Transgenic plants showing correct tissue-specific expression of GUS-SPA1, were then analyzed for complementation of *spa* mutant phenotypes.

ii) Functional conservation of COP1 and SPA proteins across plant species: The function of COP1 and SPA proteins is not known in plant species other than the dicot Arabidopsis. Therefore, to investigate the evolution of *COP1* and *SPA* gene functions, I asked whether COP1 and SPA proteins from the moss Physcomitrella and from the monocot rice are functional in Arabidopsis. To this end, the open reading frames of rice and Physcomitrella *COP1* and *SPA* homologs were expressed in Arabidopsis *cop1* and *spa* mutant plants, respectively, and transgenic plants were then analyzed for complementation of the respective mutant phenotypes. This approach complements a collaborative approach, in which *SPA* genes are knocked out in Physcomitrella to investigate SPA functions in this moss.

III. Results

III. 1. Cell-cell communication in SPA1-regulated plant development

Intercellular/interorgan communications in light signaling have been well documented by now. However, very little is known about the underlying mechanisms of long-distance communication in light-regulated plant development. Recent studies demonstrated that the light signal perception in leaves and cotyledons may regulate the developmental processes in distant organs via long distance transport of protein molecules as seen for photoperiodic flowering or via the modulation of hormone signaling as seen for elongation responses. Moreover, except for photoperiodic flowering, the tissue-specific responses in light signaling are mostly characterized for photoreceptors, but tissue-specific responses of genes downstream of these photoreceptors are largely unknown. Recent gene expression studies showed organ-specific light dependent gene expression in Arabidopsis, suggesting a possible involvement of components downstream of the photoreceptors in tissue-specific light signaling pathways (Jiao et al., 2005). The COP1/SPA complex is a very crucial component of light signal transduction, linking photoreceptors to various transcription factors involved in light signaling. Therefore, investigation of the functional sites of the COP1/SPA complex to regulate different stages of plant development should provide a deeper insight into the role of cell-cell communication in light-regulated plant development.

III. 1. 1. Spatial pattern of GUS-SPA1 accumulation in Arabidopsis

Previously *SPA1* has been shown to express at seedling stage in both light and darkness as well as in adult plants through transcript analysis (Fittinghoff et al., 2006). In the present study, I first investigated the spatial expression pattern of SPA1 protein in both seedling and adult plant stage of Arabidopsis. To this end, a β-glucuronidase(GUS)-SPA1 fusion protein was expressed under the control of the native *SPA1* promoter (*ProSPA1:GUS-SPA1*) in transgenic *spa* mutant plants (*spa1 spa2 spa3* and *spa1 spa3 spa4*). These two *spa* mutant backgrounds were used because *spa1 spa2 spa3* exhibits strong photomorphogenesis in dark-grown seedlings, while *spa1 spa3 spa4* shows a strong mutant phenotype at the adult stage (Laubinger et al., 2004; Fittinghoff et al., 2006). Thus, no *spa* triple mutant is suitable to analyze both, seedling and adult phenotypes. GUS was used as a reporter gene, rather than GFP, because of its higher sensitivity in detecting low-level expression at tissue resolution (de Ruijter et al., 2003). Transgenic lines expressing *ProSPA1:GUS-SPA1* in the *spa1 spa2 spa3*

triple mutant showed full complementation of the *spa* mutant seedling phenotype, whereas transgenic lines expressing *ProSPA1:GUS-SPA1* in the *spa1 spa3 spa4* triple mutant showed full complementation of *spa* mutant phenotypes at the adult plant stage (see later also), indicating that the transgene was fully functional. At least 25 independent transgenic lines were isolated in each triple mutant background. The analyses of spatial patterns of GUS-SPA1 levels in a representative transgenic line in the *spa1 spa2 spa3* and the *spa1 spa3 spa4* mutant are shown in Figure 7 and 8, respectively. Supplemental figures S1 and S2, further, show spatial accumulation of GUS-SPA1 in two additional homozygous transgenic lines in each triple mutant background.

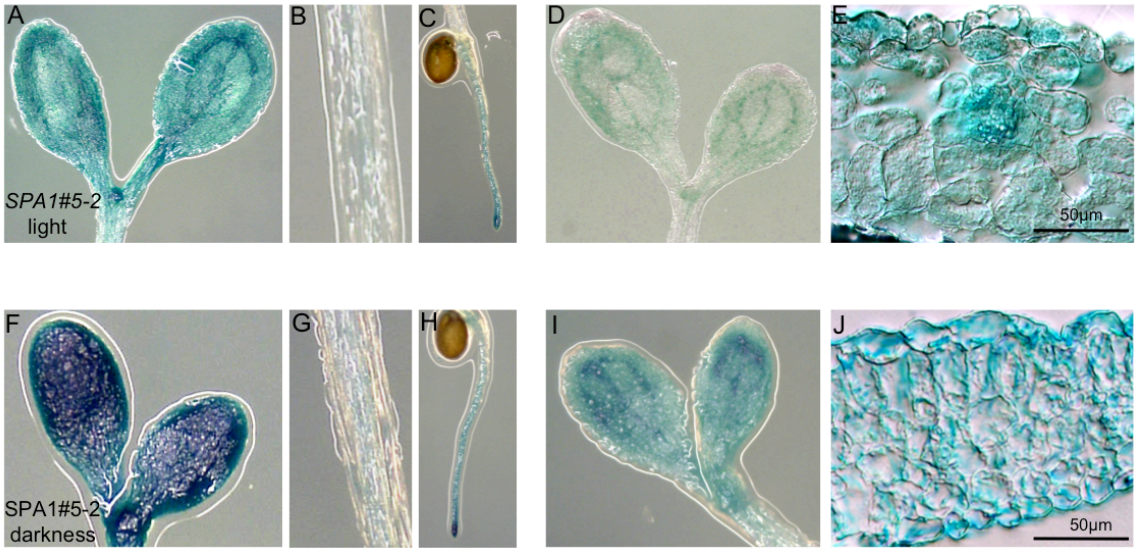

Figure 7: GUS-SPA1 accumulation in seedlings of a representative homozygous transgenic *spa1 spa2 spa3* mutant expressing *GUS-SPA1* under the control of the native *SPA1* promoter (*SPA1:GUS-SPA1#5-2*).

(A) – **(E)** GUS-staining pattern in four-day-old red-light-grown (0.1 µmol m^{-2} s^{-1}) transgenic seedlings. Cotyledons (A), the hypocotyl (B) and the root (C) of a transgenic seedling stained overnight for GUS activity; Cotyledons after 4 hours of GUS-staining (D); Cross-section of an overnight GUS-stained cotyledon (E).
(F) – **(J)** GUS-staining pattern in four-day-old dark-grown transgenic seedlings. Cotyledons (F), the hypocotyl (G) and the root (H) of a transgenic seedling stained overnight for GUS activity; Cotyledons after 4 hours of GUS-staining (I); Cross-section of an overnight GUS-stained cotyledon (J).

GUS-SPA1 accumulated in all the three major organs of the seedling i.e. in cotyledons, the hypocotyl and the root in both light (Figure 7A, B, C) and darkness (Figure 7F, G, H). GUS activity was higher in cotyledons and roots when compared to hypocotyls. Interestingly, we observed that GUS-SPA1 accumulated to higher levels in dark-grown seedlings than in light-grown seedlings (Figure 7A, F). Shorter GUS-staining revealed that GUS-SPA1 was very strongly expressed in vascular bundles in both light

and darkness (Figure 7D, I). Cross sections through cotyledons of transgenic seedlings expressing *ProSPA1:GUS-SPA1* revealed that GUS-SPA1 accumulated in the epidermis, the mesophyll and in vascular bundles in both light and darkness (Figures 7E, J).

Also in adult plants, GUS-SPA1 accumulated ubiquitously. GUS activity was detected in leaves, the shoot apex, the stem and the root (Figure 8A). GUS-SPA1 accumulated in all the three major radial tissues i.e. the epidermis, the leaf mesophyll and in vascular bundles (Figure 8B). The level of GUS-SPA1 activity varied among the three tissue layers, with very strong expression in vascular bundles and low level of expression in the epidermis (Figures 8B, C). In stem vascular bundles, GUS-SPA1 was exclusively expressed in the phloem (both sieve tube and companion cells, Figure 8D).

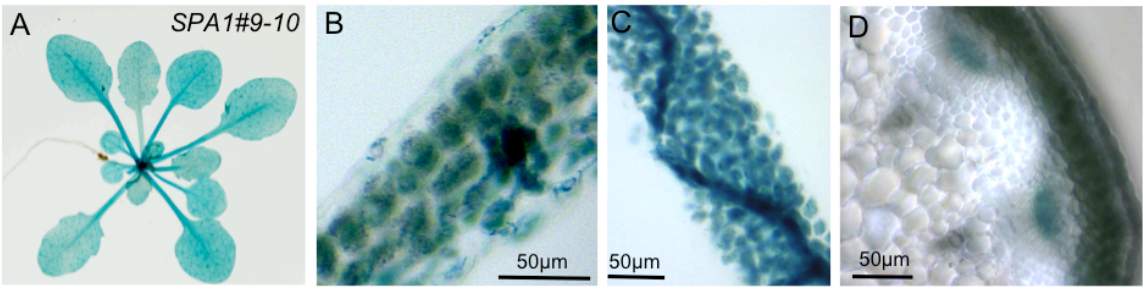

Figure 8: GUS-staining pattern in a representative homozygous transgenic *spa1 spa3 spa4* plant expressing *GUS-SPA1* under the control of the native *SPA1* promoter (*SPA1:GUS-SPA1#9-10*).

(A) A 3-week-old long-day-grown transgenic plant.
(B) and **(C)** Free-hand cross-sections through leaves of transgenic plants.
(D) Free-hand cross-section of the stem of a transgenic plant.

III. 1. 2. Tissue-specific expression of GUS-SPA1 in transgenic *spa1 spa3 spa4* plants

Cell-cell communication in light-regulated plant development is known at both the seedling and the adult plant stage. For example, light perception in the cotyledons regulates hypocotyl elongation at the seedling stage (Endo et al., 2005; Warnasooriya et al., 2009). Similarly, photoreceptors function in leaf tissues to regulate photoperiodic flowering at the shoot apical meristem (Endo et al., 2005; 2007). SPA proteins regulate plant developmental processes right from the seedling stage through to the adult plant stage and flower induction (Laubinger et al, 2004; 2006). Therefore, I aimed to investigate the functional sites of SPA1 protein in regulation of these plant developmental processes.

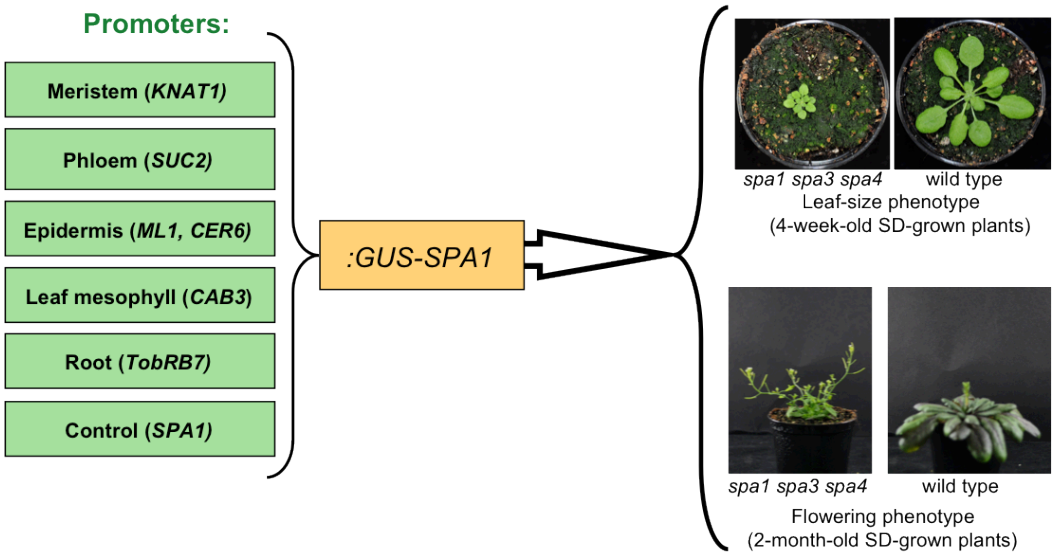

Figure 9: Strategy to study the functional sites of SPA1 to regulate leaf size and flowering time.

A *GUS-SPA1* fusion was expressed under the control of different tissue-specific promoters as well as under the native *SPA1* promoter in the *spa1 spa3 spa4* mutant and transgenic triple mutant plants expressing GUS-SPA1 in specific tissues were analyzed for the complementation of leaf size and flowering time phenotypes.

spa1 spa3 spa4 mutant plants are very small in size and show extremely early flowering in short days when compared to wild-type plants (Laubinger et al., 2004 and Laubinger et al., 2006). In order to investigate in which tissues SPA1 acts to regulate photoperiodic flowering and leaf size, a GUS-SPA1 fusion protein was expressed under the control of different tissue-specifically expressed promoters in the *spa1 spa3 spa4* triple mutant (Figure 9). Promoters used to drive tissue-specific expression of *GUS-SPA1* were $SUC2_{pro}$ for phloem specificity (Truernit and Sauer, 1995; Stadler and Sauer, 1996), $CAB3_{pro}$ for mesophyll specificity (Susek et al., 1993), $ML1_{pro}$ (Lu et al., 1996; Sessions et al., 1999) and $CER6_{pro}$ (Hooker et al., 2002) for epidermis specificity, $KNAT1_{pro}$ for shoot meristem specificity (Lincoln et el., 1994) and $TobRB7_{pro}$ for root specificity (Yamamoto et al., 1991). Previously, all these promoters have been successfully used to study the spatial regulation of photoperiodic flowering through CO and cry2 (An et al., 2004; Endo et al., 2007).

For each promoter construct, approximately 50 independent T1 lines were screened to identify lines showing correct tissue-specific expression of the transgene. These lines were subsequently used for phenotypic analysis. Transgenic lines carrying *ProSUC2:GUS-SPA1* showed expression exclusively in vascular bundles. Fully developed leaves of these lines showed strong vascular bundle-specific expression of GUS-SPA1 (Figure 10A). In developing leaves, the phloem-specific expression level was lower but well detectable (Figure 10B). Very young leaves (~ 2mm in length) showed

faint phloem-specific GUS-SPA1 expression, usually limited to the leaf tip (Figure 10C). Initiating leaf primordia did not show detectable GUS-SPA1 expression (data not shown). The observed differences in the level of GUS-SPA1 at different stages of leaf development when expressed under the *SUC2* promoter were consistent with the earlier characterization of the promoter (Truernit and Sauer, 1995; Imlau et al., 1999). Cross sections through leaves of *SUC2:GUS-SPA1* transgenic lines confirmed that GUS-SPA1 expression was restricted to vascular bundles (Figure 10D, E).

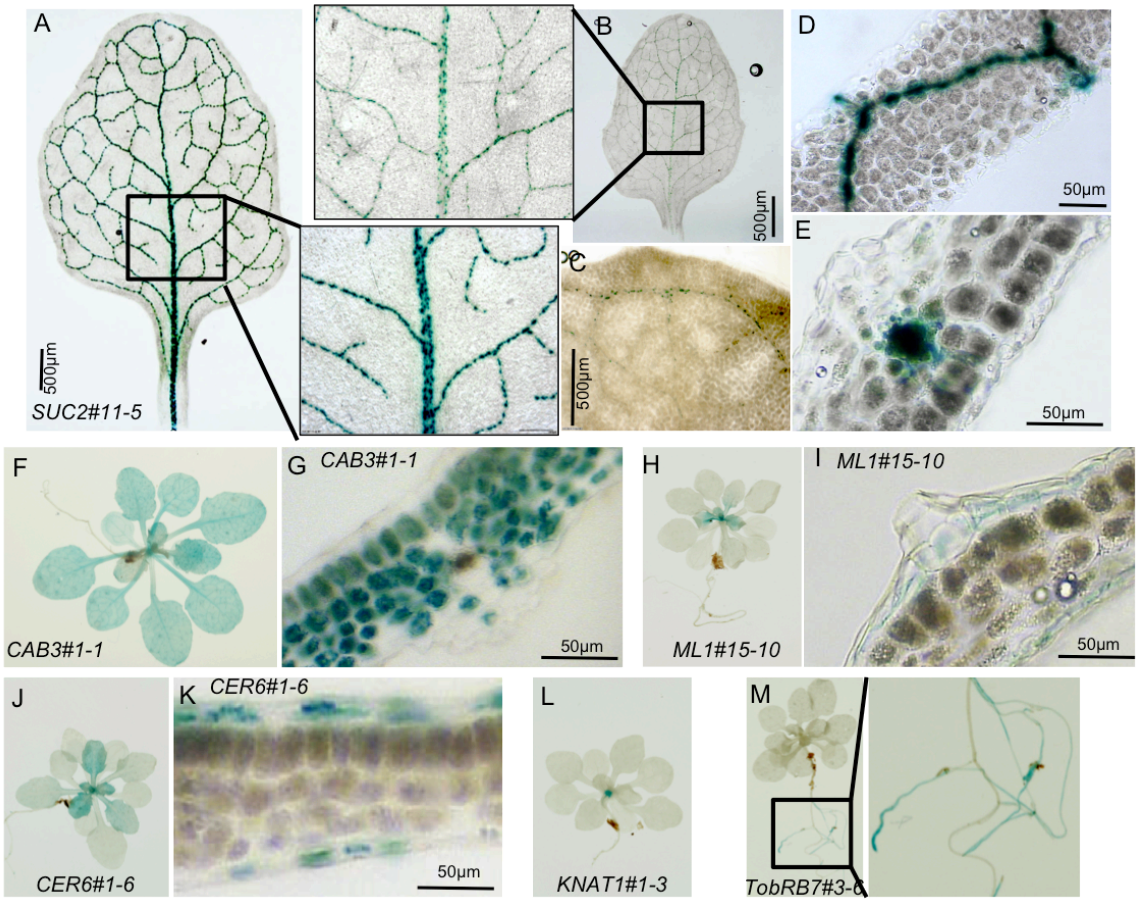

Figure 10: Tissue-specific expression of GUS-SPA1 in representative homozygous transgenic *spa1 spa3 spa4* plants expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters.

(A) – **(C)** Matured (A), developing (B) and young (C) leaves of a transgenic *SUC2:GUS-SPA1#11-5* plant.
(D) – **(E)** Free-hand cross-sections through leaves of *SUC2:GUS-SPA1#11-5* transgenic plants.
(F) – **(G)** A 3-week-old transgenic *CAB3:GUS-SPA1#1-1* plant (F) and cross section through a leaf (G) of the transgenic plant.
(H) – **(I)** A 3-week-old transgenic *ML1:GUS-SPA1#15-10* plant (H) and cross section through a leaf (I) of the transgenic plant.
(J) – **(K)** A 3-week-old transgenic *CER6:GUS-SPA1#1-6* plant (J) and cross section through a leaf (K) of the transgenic plant.
(L) A 3-week-old transgenic *KNAT1:GUS-SPA1#1-3* plant.
(M) A 3-week-old transgenic *TobRB7:GUS-SPA1#3-6* plant.

ProCAB3:GUS-SPA1 transgenic lines showed GUS-SPA1 expression restricted to the leaf mesophyll in young and fully developed leaves. No GUS-SPA1 signal was detectable in the vascular bundle or in the epidermis (Figure 10F,G). Exclusive expression of GUS-SPA1 in the epidermis was detected in *ProML1:GUS-SPA1* (Figure 10H, I) and *ProCER6:GUS-SPA1* (Figure 10J, K) transgenic lines. The expression level with both epidermis-specifically expressed promoters was strong in very young developing leaves, leaf primordia and the shoot apex and decreased to very low levels in fully developed leaves. Some of the *ML1:GUS-SPA1* transgenic lines showed very low levels of GUS-SPA1 in sub-epidermal layers (data not shown), although none of those lines showing expression in the sub-epidermis was used for final phenotypic analysis. The observed expression pattern of the *ML1* promoter is consistent with earlier characterizations of the promoter (Lu et al., 1996; Sessions et al., 1999). *ProKNAT1:GUS-SPA1* and *ProTobRB7:GUS-SPA1* transgenic lines were also confirmed to show shoot meristem- and root-specific expression of GUS-SPA1, respectively (Figure 10L, M).

The levels of GUS-SPA1 in different tissues under the control of respective tissue-specifically expressed promoters were either similar to or higher than those of GUS-SPA1 under the control of the native *SPA1* promoter in respective tissues. The native *SPA1* promoter led to strong accumulation of GUS-SPA1 in phloem and mesophyll tissues and to low levels in the epidermis (Figure 8B). The *SUC2* promoter also conferred strong accumulation of GUS-SPA1 in the phloem (Figure 10D, E). The *CAB3* promoter, similar to the native SPA1 promoter, led to high levels of GUS-SPA1 in the mesophyll (Figure 10G). *ML1* and *CER6* promoters caused accumulation of GUS-SPA1 in the epidermis to levels higher than or similar to those observed in lines expressing the GUS-SPA1 under the native *SPA1* promoter (Figure 10I, K). Similarly, *KNAT1* and *TobRB7* promoters led to similar or higher levels of GUS-SPA1 in the shoot apical meristem and the root, respectively, than the *ProSPA1:GUS-SPA1* transgenic lines (Figure 10L, M).

For each construct, three independent representative homozygous transgenic lines showing correct tissue-specific expression of GUS-SPA1 were propagated to obtain homozygous transgenic plants. These were subsequently used for detailed phenotypic analysis. Transgenic lines expressing GUS-SPA1 under the control of the native *SPA1* promoter were used as controls. Supplemental figures S3 and S4 show tissue-specific expression of GUS-SPA1 in two additional homozygous transgenic lines expressing GUS-SPA1 under the control of each tissue-specifically expressed promoter.

III. 1. 3. SPA1 acts in the phloem to regulate photoperiodic flowering

SPA proteins are essential for photoperiodic flowering because they repress flowering under non-inductive short day conditions (Laubinger et al., 2006). Photoreceptors and CO have already been shown to act in specific tissues to regulate photoperiodic flowering in Arabidopsis (An et al., 2004; Endo et al., 2005 and Endo et al., 2007). Therefore, I first examined in which tissues SPA1 acts to regulate this process.

The *spa1 spa3 spa4* mutant shows complete loss of photoperiodic control of flowering. It flowers very early in short days and slightly early in long days when compared to the wild type (Figure 11, Laubinger et al., 2006). The *spa3 spa4* mutant flowers as late as the wild type in both short and long days (Figure 11), indicating that SPA1 is necessary and sufficient to regulate photoperiodic flowering. Indeed, expression of *GUS-SPA1* under the control of the native *SPA1* promoter led to full complementation of the early-flowering phenotype of the *spa1 spa3 spa4* triple mutant in both short and

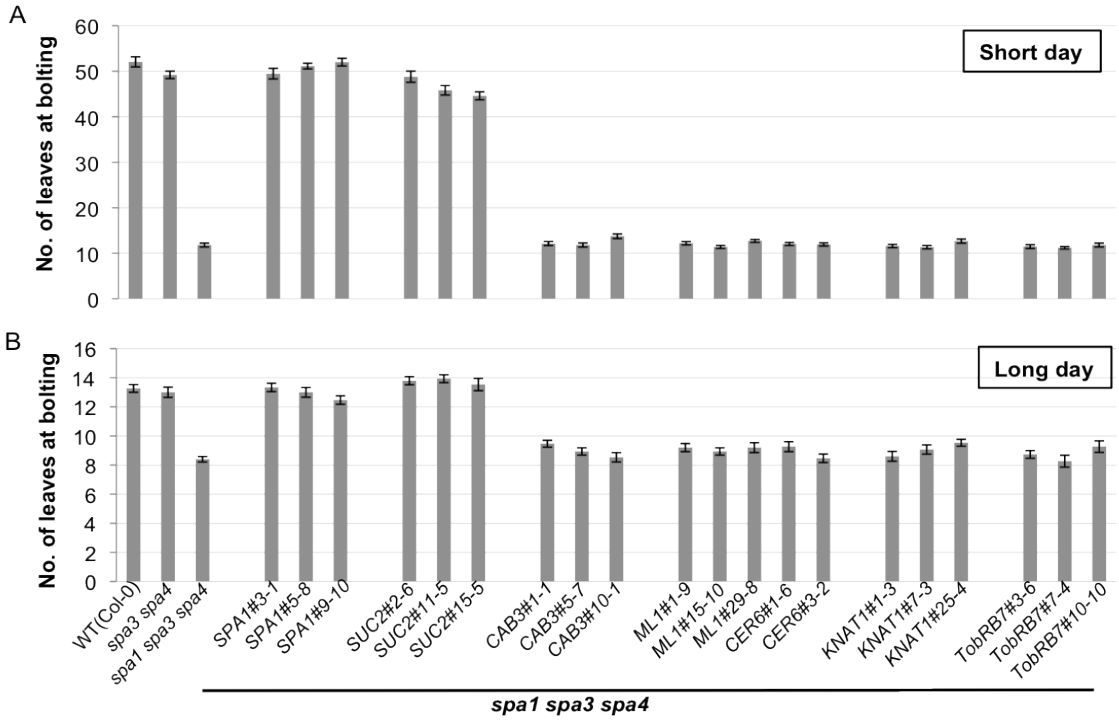

Figure 11: SPA1 acts in the phloem to regulate flowering time.

(A) and **(B)** Quantification of flowering time of homozygous transgenic plants expressing GUS-SPA1 in specific tissues in the *spa1 spa3 spa4* mutant in short days (A) and long days (B). For each transgene, two to three independent transgenic lines were analyzed. Transgenic lines are denoted as the promoters used to drive the *GUS-SPA1* expression. As controls, flowering time of wild-type (WT), *spa3 spa4* and *spa1 spa3 spa4* mutant plants were quantified. Plants were grown in short days (8 hour light/16hour dark) or long days (16 hour light/8 hour dark) and flowering time was determined by counting the number of rosette leaves at flowering. At least 15 plants were analyzed per genotype. Error bars denote the standard error of the mean.

long days (Figure 11A, B; Supplemental figure S5). Similarly, *ProSUC2:GUS-SPA1* transgenic lines, expressing GUS-SPA1 exclusively in the phloem, showed full complementation of the early-flowering phenotype of the parental *spa* triple mutant. These transgenic lines flowered as late as the wild type, the *spa3 spa4* mutant or the *ProSPA1:GUS-SPA1* transgenic lines. In contrast, mesophyll- or meristem-specific expression of *GUS-SPA1* under the control of *CAB3* and *KNAT1* promoters, respectively, did not rescue the early-flowering phenotype of the *spa* triple mutant. Similarly, *ProML1:GUS-SPA1/ProCER6:GUS-SPA1* and *ProTobRB7:GUS-SPA1* transgenic lines expressing *GUS-SPA1* in epidermis and root tissues, respectively, failed to complement the *spa* mutant phenotype (Figure 11A, B; Supplemental figure S5). In summary, these data demonstrate that SPA1 acts exclusively in the phloem to control photoperiodic flowering.

III. 1. 4. SPA1 acts in phloem and mesophyll tissues to regulate leaf size

spa1 spa3 spa4 triple mutant plants are very small in size when compared to wild-type plants. The *spa3 spa4* double mutant, with functional *SPA1*, has larger plant and leaf size than the *spa1 spa3 spa4* mutant (Figure 12). This indicates that *SPA1* plays a significant role in the regulation of adult plant and leaf size (Laubinger et al., 2004; Fittinghoff et al., 2006). In order to identify the tissues in which SPA1 regulates leaf size, I analyzed this phenotype in homozygous transgenic *spa1 spa3 spa4* plants expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters in both short and long days (Figure 12; Supplemental figures S6, S7). These were the same lines as those used for the analysis of flowering time (III. 1. 3).

As expected, *GUS-SPA1* expression under the control of the *SPA1* native promoter fully complemented the leaf-size phenotype of the parental *spa* triple mutant in long and short days (Figure 12; Supplemental figures S6, S7). Phloem-specific expression of *GUS-SPA1* under the control of the *SUC2* promoter partially complemented the leaf-size phenotype of the *spa* triple mutant. Leaves of these transgenic plants were larger than those of the parental *spa* triple mutant, but smaller than those of the *spa3 spa4* double mutant. *ProCAB3:GUS-SPA1* transgenic plants expressing *GUS-SPA1* exclusively in the mesophyll also showed an increase in leaf size when compared to the *spa* triple mutant progenitor. However, leaves of these transgenic plants were also smaller than those of the *spa3 spa4* double mutant. In contrast, epidermis-specific expression of *GUS-SPA1* under the control of the *ML1* or *CER6* promoters did not affect the leaf size of the *spa* triple mutant. *ProKNAT1:GUS-SPA1* and *ProTobRB7:GUS-SPA1* transgenic lines expressing *GUS-SPA1* in the shoot apical

meristem and the root, respectively, also failed to complement the leaf-size phenotype of the parental *spa* triple mutant (Figure 12; Supplemental figures S6, S7). Taken together, these data indicate that SPA1 acts in both the phloem and the leaf mesophyll, but not in the epidermis, to regulate leaf size.

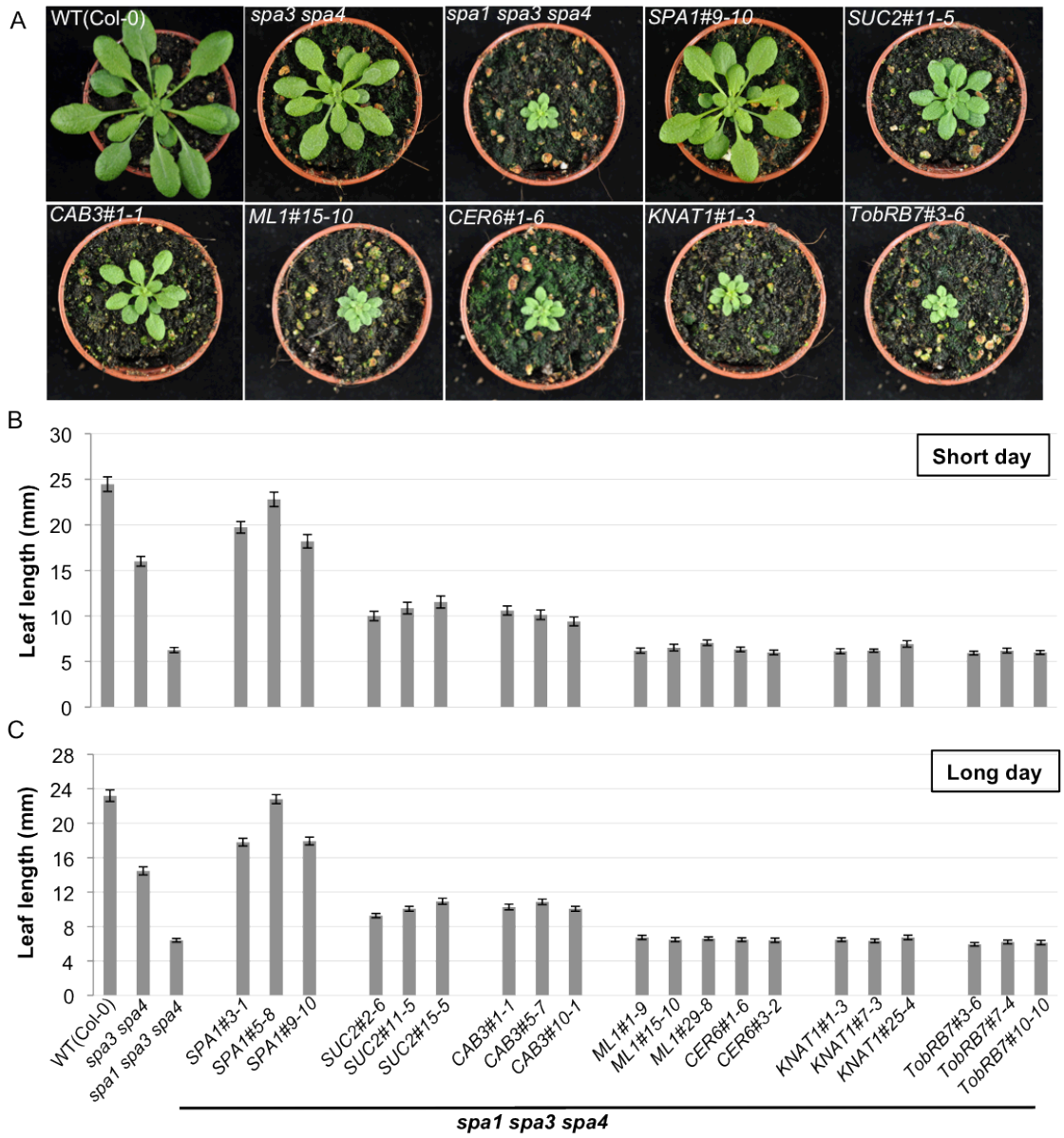

Figure 12: SPA1 acts in both the phloem and the mesophyll to regulate plant and leaf size.

(A) Visual phenotype of representative homozygous transgenic *spa1 spa3 spa4* mutant plants expressing GUS-SPA1 in specific tissues. Plants were grown in short days for four weeks. One representative homozygous transgenic plant for each transgene is shown. Transgenic plants are denoted as the promoters used to drive the *GUS-SPA1* expression. As controls, wild-type (WT), *spa3 spa4* and *spa1 spa3 spa4* mutant plants are shown.
(B) and **(C)** Quantification of leaf length in short days (B) and long days (C). For each transgene, two to three independent transgenic lines were quantified. As controls, leaf size of wild-type (WT), *spa3 spa4* and *spa1 spa3 spa4* mutant plants were quantified. The length of the biggest leaf was measured in 4-week-old short-day-grown plants or 3-week-old long-day-grown plants. At least 15 plants were analyzed per genotype. Error bars denote the standard error of the mean.

In order to examine the interaction between SPA1 activity in the phloem and in the mesophyll for leaf size regulation, I crossed homozygous transgenic plants harboring *CAB3:GUS-SPA1* and *SUC2:GUS-SPA1* constructs and used the resultant F1 double transgenic plants to analyze the leaf phenotype. Interestingly, double transgenic plants, expressing GUS-SPA1 in both the mesophyll and the phloem, fully complemented the leaf size phenotype of the parental *spa* triple mutant (Figure 13A, B). These double transgenic plants were larger than the parental single transgenic plants and appeared similar to the *spa3 spa4* double mutant. This indicates that SPA1 expression in both phloem and mesophyll tissues are necessary for normal regulation of leaf size.

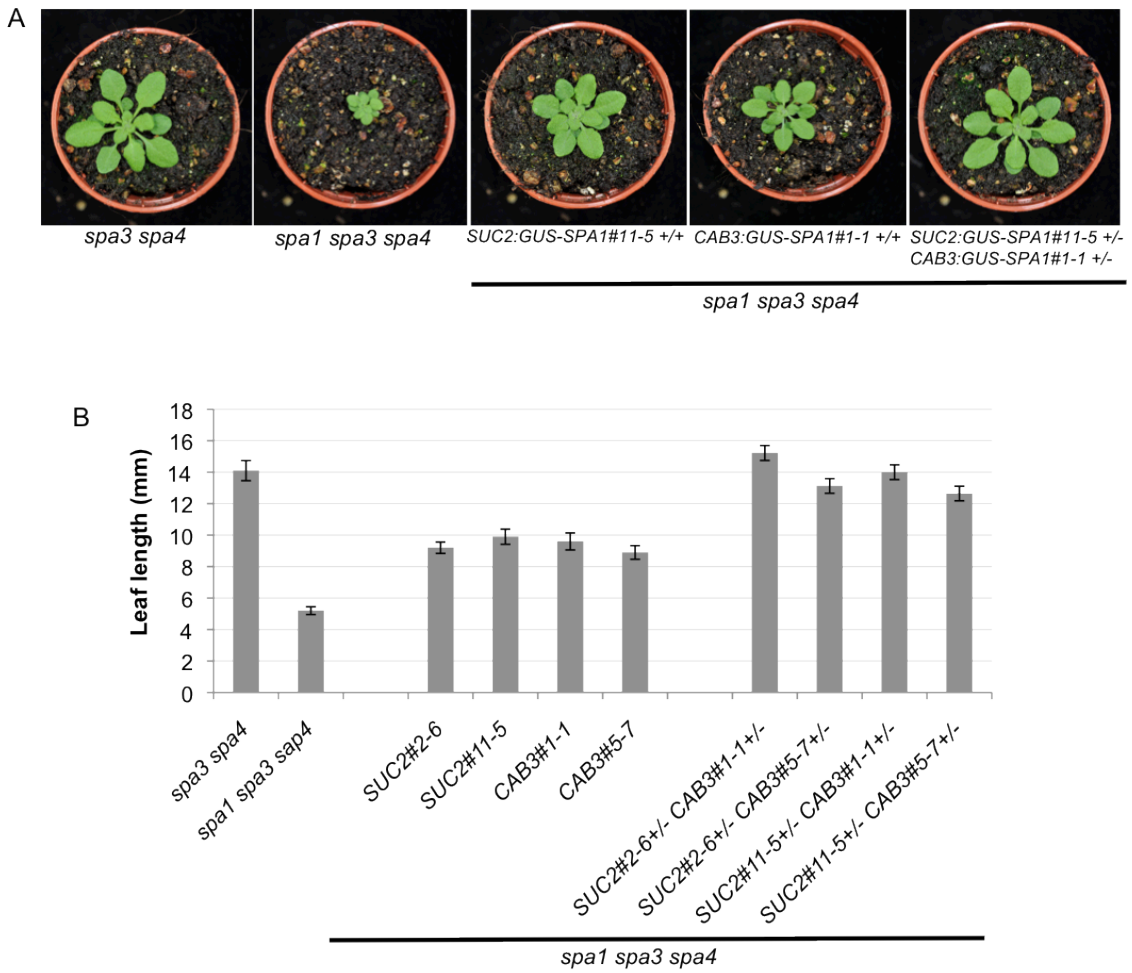

Figure 13: SPA1 activity in the phloem and the mesophyll has additive effects on leaf size.

(A) Visual phenotype of representative transgenic *SUC2:GUS-SPA1#2-6*, *CAB3:GUS-SPA1#1-1* plants and a double transgenic plant. The double transgenic plant (F1) is hemizygous for both transgenes. Plants were grown in short days for four weeks. As controls, *spa3 spa4* and *spa1 spa3 spa4* mutant plants are shown.
(B) Quantification of leaf length in short days. Shown are the leaf sizes of two independent homozygous lines for each *SUC2* and *CAB3* promoters expressing *GUS-SPA1* in the *spa1 spa3 spa4* mutant and F1 double transgenic lines resulting from the crosses of the homozygous transgenic lines. As controls, *spa3 spa4* and *spa1 spa3 spa4* mutant plants were quantified. Plants were grown in short days for four weeks. Error bars denote the standard error of the mean.

III. 1. 5. Genetic interaction of *SPA1* and photoreceptors in the regulation of photoperiodic flowering and leaf size

The photoreceptors phyB and cry2 have previously been shown to regulate photoperiodic flowering in a non-cell autonomous fashion. cry2 acts in the phloem, whereas phyB acts in the mesophyll to regulate flowering time (Endo et al., 2005; 2007). Additionally, phyB has been suggested to act in the mesophyll to regulate rosette leaf morphology (Endo et al., 2005).

In order to investigate the epistatic relationship between SPA1-regulated and photoreceptor-regulated photoperiodic flowering, I examined the flowering-time phenotype of *spa1 cry2* and *spa1 phyB* mutants in comparison to the respective single mutants and the wild type. Additionally, the leaf morphology of the *spa1 phyB* mutant in comparison to the respective single mutants and the wild type was analyzed. Figure 14A shows that the *spa1* mutant flowered earlier than the wild-type, whereas the *cry2* mutant flowered as late as the wild type in short days. The *spa1 cry2* mutant flowered as early as the *spa1* single mutant, indicating that *spa1* is fully epistatic to *cry2* in photoperiodic flowering. This suggests that *SPA1* acts genetically downstream of *CRY2* in the same pathway to regulate photoperiodic flowering.

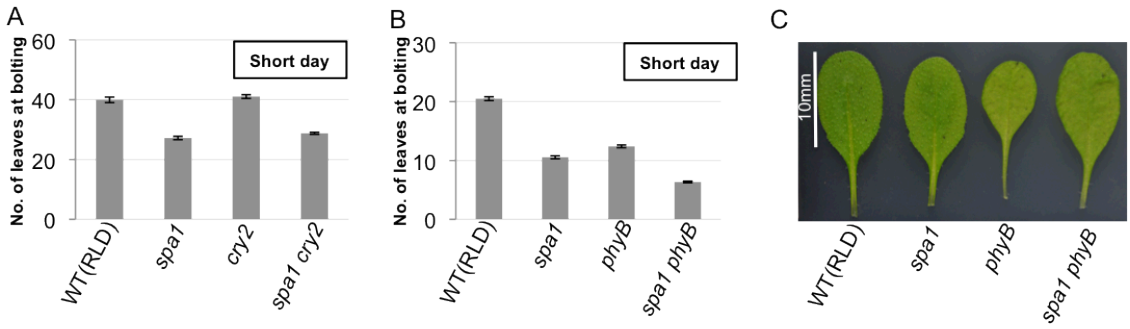

Figure 14: Genetic interaction of *spa1* with *cry2* and *phyB* mutations.

(A) Quantification of flowering time of wild-type (WT), *spa1*, *cry2* and *spa1 cry2* mutant plants in short days. Error bars denote the standard error of the mean.
(B) Quantification of flowering time of wild-type (WT), *spa1*, *phyB* and *spa1 phyB* mutant plants in short days. Error bars denote the standard error of the mean.
(C) Leaf morphology of wild-type, *spa1*, *phyB* and *spa1 phyB* mutant plants grown in short days for three weeks.

Both *spa1* and *phyB* single mutant plants flowered early in short days when compared to wild-type plants. Interestingly, the *spa1 phyB* mutant flowered even earlier than both single mutant plants (Figure 14B), indicating that *spa1* and *phyB* mutations have additive effects in photoperiodic flowering. This suggests that *SPA1* and *PHYB* act in independent pathways to regulate this process.

The *phyB* mutant has a strikingly different leaf morphology when compared to the wild type. Leaves of the *phyB* mutant show constitutive shade-avoidance, displaying smaller leaf blades and longer petioles when compared to leaves of wild-type plants (Reed et al., 1993). Leaves of the *spa1* mutant appear similar to those of the wild-type (Laubinger et al., 2004). Interestingly, leaves of the *spa1 phyB* double mutant did not exhibit a constitutive shade avoidance response (Figure 14C). They appeared similar to wild-type and *spa1* mutant leaves. This indicates that the *phyB* mutation requires the presence of functional SPA1 to show the striking leaf phenotype and that *spa1* is epistatic to *phyB* in the regulation of leaf morphology. Thus, SPA1 acts downstream of phyB in this process.

III. 1. 6. Tissue-specific expression of GUS-SPA1 in transgenic *spa1 spa2 spa3* seedlings

SPA1 and *SPA2* are sufficient for seedling skotomorhogenesis. Due to presence of functional *SPA2,* the *spa1 spa3 spa4* mutant that was used for studying adult plant traits shows a seedling phenotype similar to the wild type in darkness (Laubinger et al., 2004). Thus, analysis of seedling skotomorphogenesis is not possible in the *spa1 spa3 spa4* triple mutant. In contrast, *spa1 spa2 spa3* mutant seedlings, having both *SPA1* and *SPA2* non-functional, show very short hypocotyls and open cotyledons in darkness, as well as smaller hypocotyl length in low fluences of red and far-red light when compared to wild-type seedlings (Laubinger et al., 2004; Fittinghoff et al., 2006). Additionally, the *spa1 spa2 spa3* triple mutant exhibits constitutive differentiation of stomata and epidermal pavement cells in darkness (Kang et al, 2009). Therefore, the *spa1 spa2 spa3* triple mutant was used to study the functional sites of SPA1 in the regulation of seedling development in darkness and light as well as stomata and pavement cell development in darkness (Figure 15). To study these developmental processes, the same tissue-specific promoters were used that were employed for studying flowering time and leaf size, except for the *CER6* promoter because *CER6* is not expressed in dark-grown seedlings (Hooker et al., 2002). Additionally, *RolC* was used as a phloem-specific promoter (Booker et al., 2003) in darkness because *SUC2* failed to confer phloem-specific expression of GUS-SPA1 in cotyledons of dark-grown seedlings (data not shown).

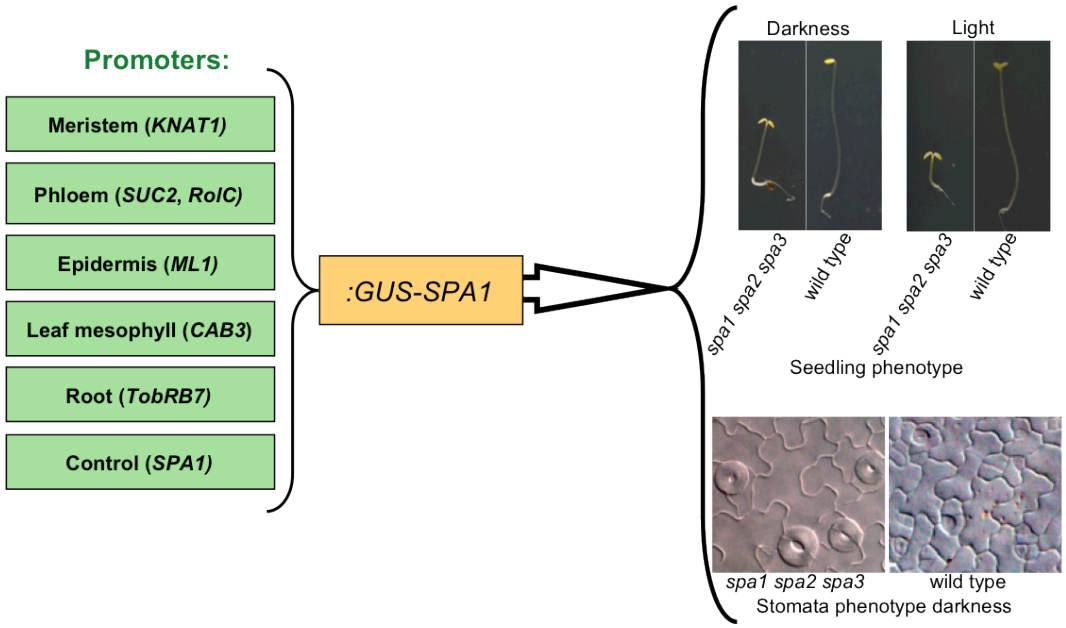

Figure 15: Strategy to study the functional sites of SPA1 to regulate seedling development and stomata differentiation.

A *GUS-SPA1* fusion was expressed under the control of different tissue-specific promoters as well as under the native *SPA1* promoter in the *spa1 spa2 spa3* mutant and transgenic triple mutant plants expressing GUS-SPA1 in specific tissues were analyzed for the complementation of the seedling phenotype in light and darkness and the stomata differentiation phenotype in darkness.

Approximately 50 independent T1 lines were screened for correct tissue-specific expression of GUS-SPA1, followed by their phenotypic analysis. The *RolC* promoter conferred phloem-specific expression of GUS-SPA1 in both dark- and light-grown transgenic seedlings (Figure 16A, B and 17C, D). Transgenic seedlings expressing GUS-SPA1 under the control of the *SUC2* promoter showed phloem-specific expression in all tissues of light-grown seedlings (Figure 17A, B) and in hypocotyls of dark-grown seedlings, but failed to confer phloem-specific expression in cotyledons of dark-grown seedlings (data not shown). The GUS-SPA1 expression level under these phloem-specific promoters was lower in the hypocotyl when compared to the cotyledons. The *SUC2* promoter led to higher levels of GUS-SPA1 than the *RolC* promoter in light-grown seedlings (Figure 17A, C). The epidermis- and mesophyll-specific expression of GUS-SPA1 in *ML1:GUS-SPA1* and *CAB3:GUS-SPA1* transgenic lines, respectively, was verified in cross-sections of cotyledons of dark- (Figure 16C to F) and light-grown transgenic seedlings (Figure 17E to H). The *ML1* promoter occasionally showed expression of GUS-SPA1 in the sub-epidermis as observed in case of young leaves. *KNAT1:GUS-SPA1* and *TobRB7:GUS-SPA1* transgenic lines showed shoot meristem- and root-specific expression of GUS-SPA1, respectively (Figure 16G-I and 17I-K). In general, all transgenic seedlings expressing GUS-SPA1 under the control of different

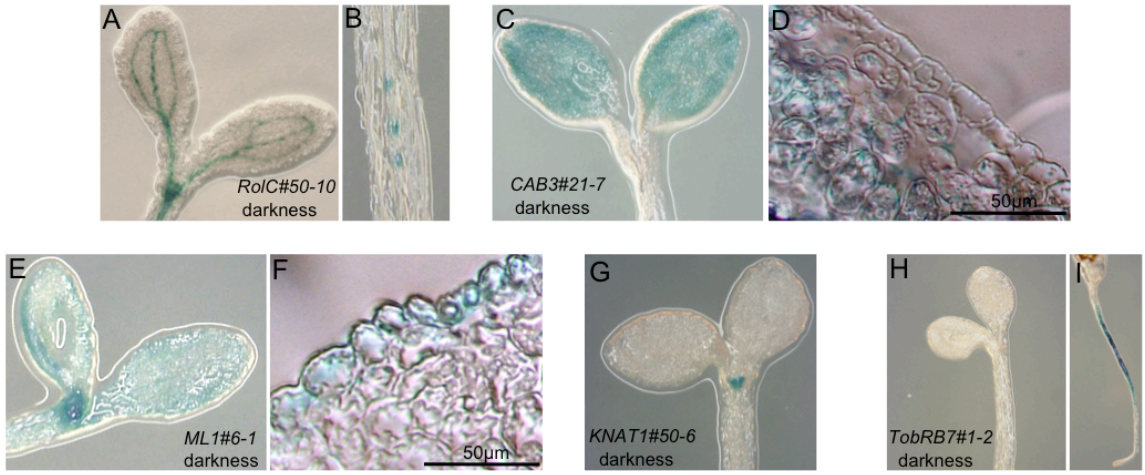

Figure 16: Tissue-specific expression of GUS-SPA1 in representative dark-grown transgenic *spa1 spa2 spa3* seedlings expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters. Seedlings were grown in darkness for four days and stained overnight for GUS activity.

(A) – **(B)** Cotyledons (A) and the hypocotyl (B) of a transgenic *RolC:GUS-SPA1#50-10* seedling.
(C) – **(D)** Cotyledons and the hypocotyl (C) and cross-section of a cotyledon (D) of a transgenic *CAB3:GUS-SPA1#21-7* seedling .
(E) – **(F**) Cotyledons and the hypocotyl (E) and cross-section of a cotyledon (F) of a transgenic *ML1:GUS-SPA1#6-1* seedling.
(G) Cotyledons and the hypocotyl of a transgenic *KNAT1:GUS-SPA1#50-6* seedling.
(H) – **(I)** Cotyledons, the hypocotyl (H) and the root (I) of a transgenic *TobRB7:GUS-SPA1#1-2* seedling.

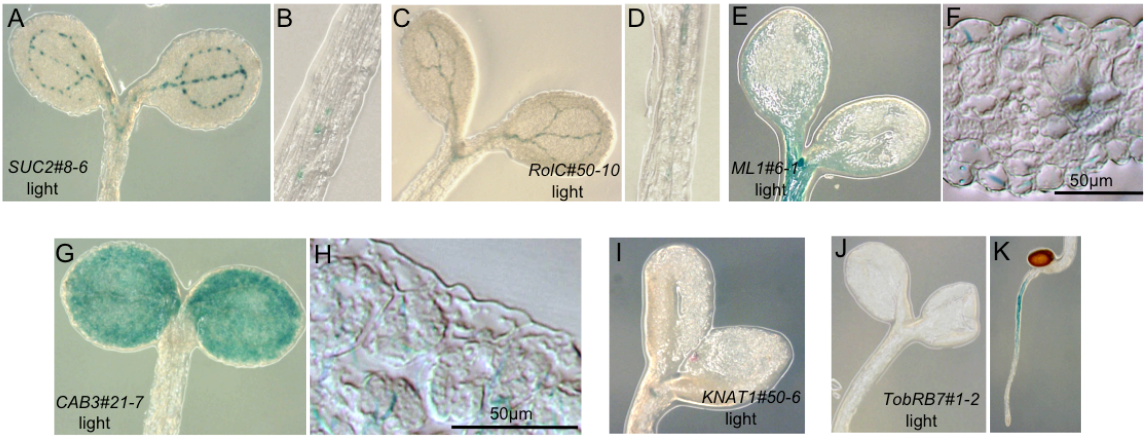

Figure 17: Tissue-specific expression of GUS-SPA1 in representative light-grown transgenic *spa1 spa2 spa3* seedlings expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters. Seedlings were grown in red-light (0.1 µmol m^{-2} s^{-1}) for four days and stained overnight for GUS activity.

(A) – **(B)** Cotyledons (A) and the hypocotyl (B) of a transgenic *SUC2:GUS-SPA1#8-6* seedling.
(C) – **(D)** Cotyledons (A) and the hypocotyl (B) of a transgenic *RolC:GUS-SPA1#50-10* seedling.
(E) – **(F)** Cotyledons and the hypocotyl (E) and cross-section of a cotyledon (F) of a transgenic *ML1:GUS-SPA1#6-1* seedling.
(G) – **(H)** Cotyledons and the hypocotyl (C) and cross-section of a cotyledon (D) of a transgenic *CAB3:GUS-SPA1#21-7* seedling.
(I) Cotyledons and the hypocotyl of a transgenic *KNAT1:GUS-SPA1#50-6* seedling.
(J) – **(K)** Cotyledons, the hypocotyl (H) and the root (I) of a transgenic *TobRB7:GUS-SPA1# 1-2* seedling.

promoters, except the *CAB3* promoter, showed lower levels of GUS-SPA1 in light-grown seedlings than in dark-grown seedlings. The expression of *CAB3* is induced by light (Kang and Ni, 2006), and hence *ProCAB3:GUS-SPA1* transgenic seedlings showed higher GUS-SPA1 accumulation in light than in dark-grown seedlings (Figure 16C, 17G).

Three independent representative homozygous transgenic lines for each construct, showing correct tissue specific expression of GUS-SPA1, were analyzed for seedling development in light and darkness as well as for stomata and pavement cell differentiation in darkness. *ProSPA1:GUS-SPA1* transgenic lines were used as controls. Supplemental figures S9 – S12 show tissue-specific expression of GUS-SPA1 in two additional homozygous transgenic *spa1 spa2 spa3* lines expressing GUS-SPA1 under the control of each tissue-specifically expressed promoter in both light and darkness.

III. 1. 7. Expression of GUS-SPA1 in the phloem regulates seedling development

Transgenic lines expressing *GUS-SPA1* under the control of the native *SPA1* promoter fully complemented the seedling phenotype of the *spa1 spa2 spa3* mutant in darkness (Figure 18A, B). These transgenic seedlings appeared similar to wild-type seedlings, confirming that SPA1 is sufficient to regulate seedling development in darkness (Fittinghoff et al., 2006). Phloem-specific expression of *GUS-SPA1* under the control of the *RolC* promoter mostly complemented the hypocotyl length phenotype of the parental *spa* triple mutant in darkness (Figure 18A, B). The transgenic seedlings displayed closed cotyledons, but no apical hook, indicating that phloem-specific expression of GUS-SPA1 has major effect on hypocotyl elongation and cotyledon closure, but no effect on apical hook formation (Figure 18A). Epidermis-specific expression of *GUS-SPA1* in *ProML1:GUS-SPA1* transgenic seedlings had only minor effects on the hypocotyl length of the *spa* triple mutant (Figure 18B). However, these transgenic seedlings displayed a partial closure of the cotyledons in darkness, whereas transgenic seedlings expressing GUS-SPA1 under other promoters showed fully open cotyledons, indicating that epidermis-specific expression of GUS-SPA1 has an effect on cotyledon closure in darkness (Figure 18A). Mesophyll-specific expression of *GUS-SPA1* under the control of the *CAB3* promoter neither complemented the hypocotyl length nor the cotyledon phenotype of the *spa* triple mutant progenitor. Meristem- or root- specific expression of *GUS-SPA1* under the control of *KNAT1* and *ML1* promoters, respectively, also did not rescue the hypocotyl length or cotyledon phenotype of the *spa* triple mutant in darkness (Figure 18A, B).

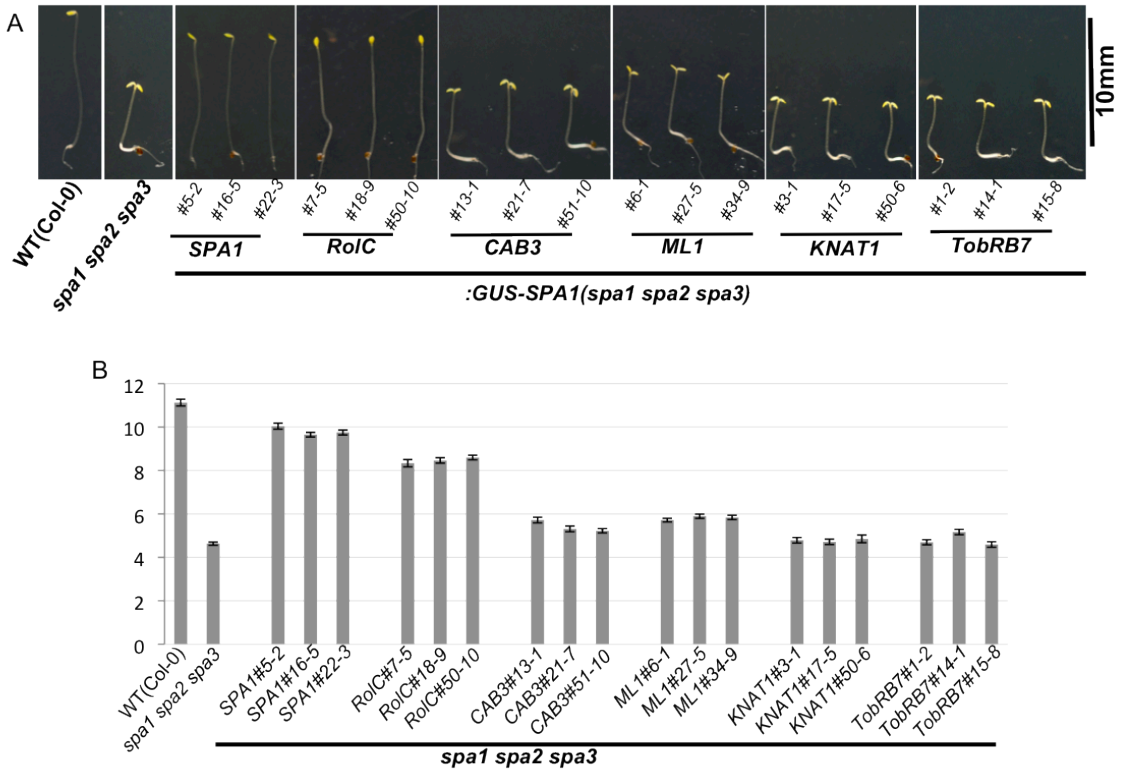

Figure 18: Phloem-specific SPA1 activity has a major contribution to seedling development in darkness.

(A) Visual phenotype of homozygous transgenic seedlings expressing *GUS-SPA1* under different promoters in the *spa1 spa2 spa3* mutant. Seedlings were grown in darkness for four days. For each transgene, three independent transgenic seedlings are shown. As controls, wild-type (WT) and *spa1 spa2 spa3* mutant seedlings are shown.
(B) Quantification of hypocotyl length of the genotypes shown in (A). Error bars denote the standard error of the mean.

Transgenic seedlings expressing GUS-SPA1 under the control of different promoters showed similar results in light-grown seedlings (19A, B). *ProSPA1:GUS-SPA1* transgenic seedlings showed full complementation of the seedling phenotype of the parental *spa* triple mutant in the light as well. Phloem-specific expression of *GUS-SPA1* under the control of both the *SUC2* and *RolC* promoters complemented the hypocotyl length phenotype of the *spa1 spa2 spa3* triple mutant in low fluences of red light (Figure 19A, B), though the effect was lower than in *ProSPA1:GUS-SPA1* transgenic lines. *GUS-SPA1* activity in the phloem under the control of the *SUC2* promoter showed a higher degree of complementation when compared to the *RolC* promoter. This was consistent with the lower level of GUS-SPA1 expression under the *RolC* promoter when compared to the *SUC2* promoter in light-grown seedlings (Figure 17A, C). Similar to darkness, mesophyll- and epidermis-specific expression of *GUS-SPA1* had only very minor effects on hypocotyl length in low fluences of red light. Neither

shoot apical meristem- nor root-specific expression of *GUS-SPA1* rescued the parental *spa* triple mutant phenotype in the light (Figure 19A, B).

Taken together, these data indicate that phloem-specific expression of SPA1 provides a major contribution to seedling development in both darkness and light. Furthermore, epidermis-specific expression of SPA1 has an effect on cotyledon closure in darkness.

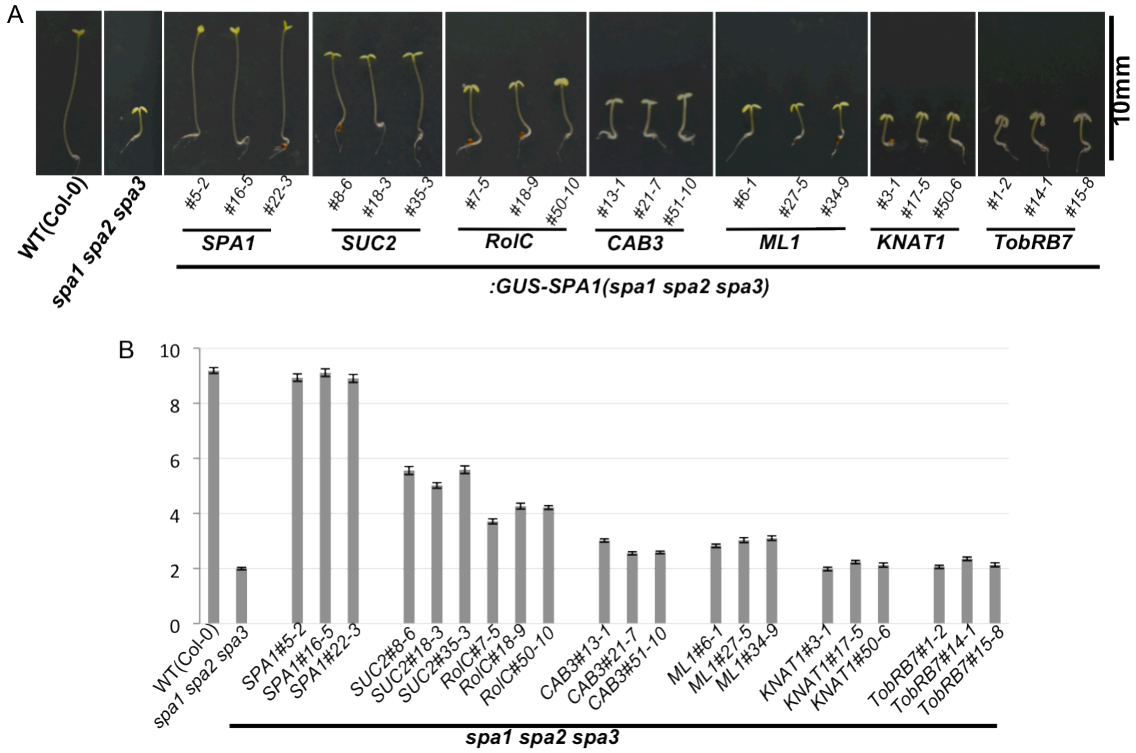

Figure 19: SPA1 activity in the phloem has a major effect on seedling development in light.

(A) Visual phenotype of homozygous transgenic seedlings expressing *GUS-SPA1* under different promoters in the *spa1 spa2 spa3* mutant. Seedlings were grown in red-light (0.1 μmol m^{-2} s^{-1}) for four days. For each transgene, three independent transgenic seedlings are shown. As controls, wild-type (WT) and *spa1 spa2 spa3* mutant seedlings are shown.
(B) Quantification of hypocotyl length of the genotypes shown in (A). Error bars denote the standard error of the mean.

III. 1. 8. SPA1 acts in the phloem to regulate stomata differentiation and epidermal pavement cell shape in darkness

Full differentiation of stomata in cotyledons requires light and is dependent on photoreceptor function (Kang et al., 2009). Dark-grown wild-type seedlings, therefore, arrest stomata development at the meristemoid stage. *cop1* and *spa1 spa2 spa3* mutants, in contrast, differentiate stomata also in darkness and thus display constitutive photomorphogenesis also with respect to this phenotype (Kang et al., 2009; Figure 22A, B). In addition, cotyledons of the *spa* triple mutant show large, multi-lobed and jigsaw puzzle-shaped epidermal pavement cells in darkness similar to wild-type seedlings

grown in light. In contrast, cotyledons of dark-grown wild-type seedlings show small, non-lobed and smooth pavement cells (Figure 20A, B). Transgenic lines expressing *GUS-SPA1* under the control of various tissue-specific promoters in the *spa1 spa2 spa3* mutant were used to examine the functional site of SPA1 in the regulation of stomata and epidermal cell development in darkness.

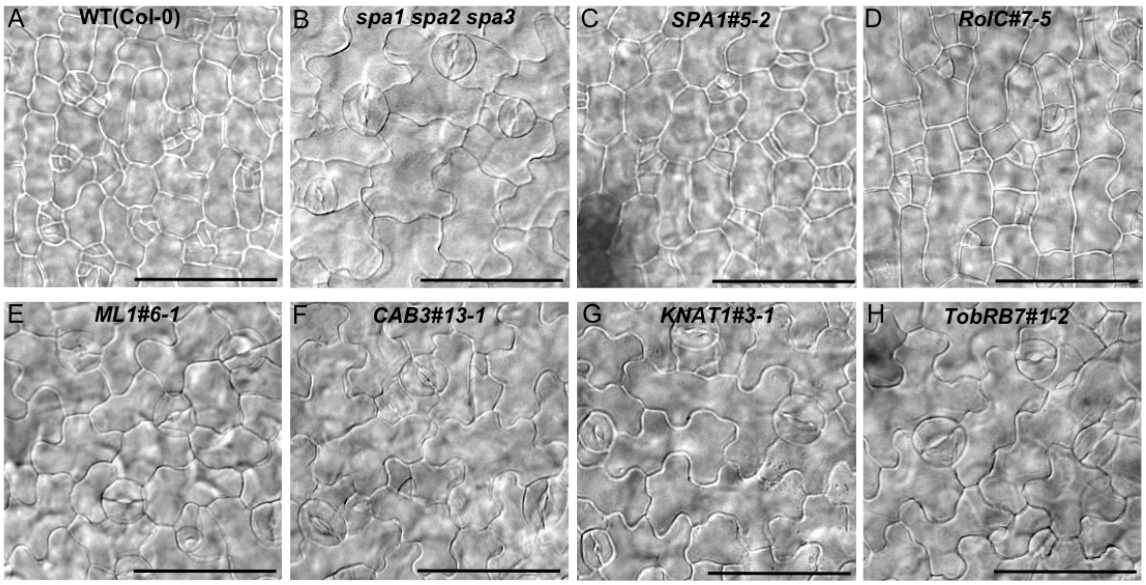

Figure 20: SPA1 acts in the phloem to regulate stomata development and epidermal pavement cell shape in darkness.

(A) to **(H)** Abaxial cotyledon epidermis of 10-day-old dark-grown wild-type(A), *spa1 spa2 spa3*(B), *SPA1:GUS-SPA1#5-2* (C), *RolC:GUS-SPA1#7-5* (D), *ML1:GUS-SPA1#6-1* (E), *CAB3:GUS-SPA1#13-1* (F), *KNAT1:GUS-SPA1#3-1* (G) and *TobRB7:GUS-SPA1#1-2* (H). All the transgenic lines are homozygous in the *spa1 spa2 spa3* triple mutant background. Scale bars = 50 μm

GUS-SPA1 expression under the control of the native *SPA1* promoter fully complemented the stomata as well as the pavement cell phenotype of the parental *spa* triple mutant (Figure 20A, B, C). These *ProSPA1:GUS-SPA1* transgenic lines showed arrested stomata development as well as non-lobed smooth epidermal cells in cotyledons of dark-grown seedlings, indicating that SPA1 is sufficient to suppress stomata differentiation and lobing of pavement cell in the dark-grown *spa1 spa2 spa3* mutant. Interestingly, *ProRolC:GUS-SPA1* lines expressing *GUS-SPA1* exclusively in the phloem also showed arrested stomata development and non-lobed smooth epidermal pavement cells in darkness (Figure 20D), similar to the cotyledons of dark-grown wild-type seedlings, with the exception that epidermal cells of cotyledons of these transgenic seedlings were larger than those of wild-type cotyledons. Epidermis-specific expression of *GUS-SPA1* under the control of the *ML1* promoter did not affect the stomata phenotype of the parental *spa* triple mutant in darkness. However, cotyledons of

dark-grown seedlings of these transgenic lines showed partial complementation of the epidermal pavement cell shape phenotype. These cotyledons exhibited reduced lobing of the epidermal cells as well as smaller epidermal cells when compared to the parental *spa* triple mutant (Figure 20E). Mesophyll-, meristem- or root- specific expression of *GUS-SPA1* through *CAB3*, *KNAT1* and *TobRB7* promoters, respectively, did not rescue the stomata or the pavement cell phenotype of the *spa* triple mutant progenitor (Figure 20F-H). Supplemental figure S13 shows stomata and pavement cell phenotype of two additional homozygous transgenic *spa1 spa2 spa3* lines expressing *GUS-SPA1* under the control of each tissue-specific promoter.

These data indicate that phloem-specific expression of SPA1 regulates stomata differentiation and epidermal pavement cell shape in darkness. Further, epidermis-specific expression of SPA1 has also some cell-autonomous effects on pavement cell shape in darkness.

III. 1. 9. Phytohormone mutants show defective stomata differentiation and pavement cell shape in darkness

Auxin-resistant mutants such as *axr2* (Nagpal et al., 2000), *axr3* (Leyser et al., 1996) and *shy2* (Reed et al., 1998) show constitutive photomorphogenesis in darkness. These auxin-resistant mutants are *iaa* gain-of-function mutants, expressing stabilized versions of IAA7, IAA17 and IAA3, respectively, that can not be degraded in the presence of auxin (Colon-Carmona et al., 2000; Gray et al., 2001; Ouellet et al., 2001). Also, the cyokinin overproducing mutant *amp1-1* (Chaudhury et al., 1993) shows constitutive photomorphogenesis in darkness (Figure 21A). I, therefore, examined stomata development and epidermal cell shape in dark-grown *iaa* and *amp1* mutants. Interestingly, cotyledons of dark-grown seedlings of all these mutants exhibited differentiated stomata, similar to the *spa1 spa2 spa3* mutant (Figure 21B-G). This indicates that auxin and cytokinin are likely involved in suppression of stomata differentiation in darkness.

Cotyledons of dark-grown seedlings of *iaa* gain-of-function auxin-resistant mutants also showed lobed epidermal pavement cells similar to the *spa1 spa2 spa3* mutant (Figure 21B-F). The extent of lobing, however, was less than that of the *spa* triple mutant. On the other hand, cotyledons of dark-grown *amp1-1* seedlings showed smooth, non-lobed pavement cells like wild-type cotyledons, although the pavement cells of *amp1-1* cotyledons were larger than those of wild type cotyledons (Figure 21B, G). These results suggest that auxin signaling is required to inhibit lobing of epidermal pavement cells in dark-grown cotyledons.

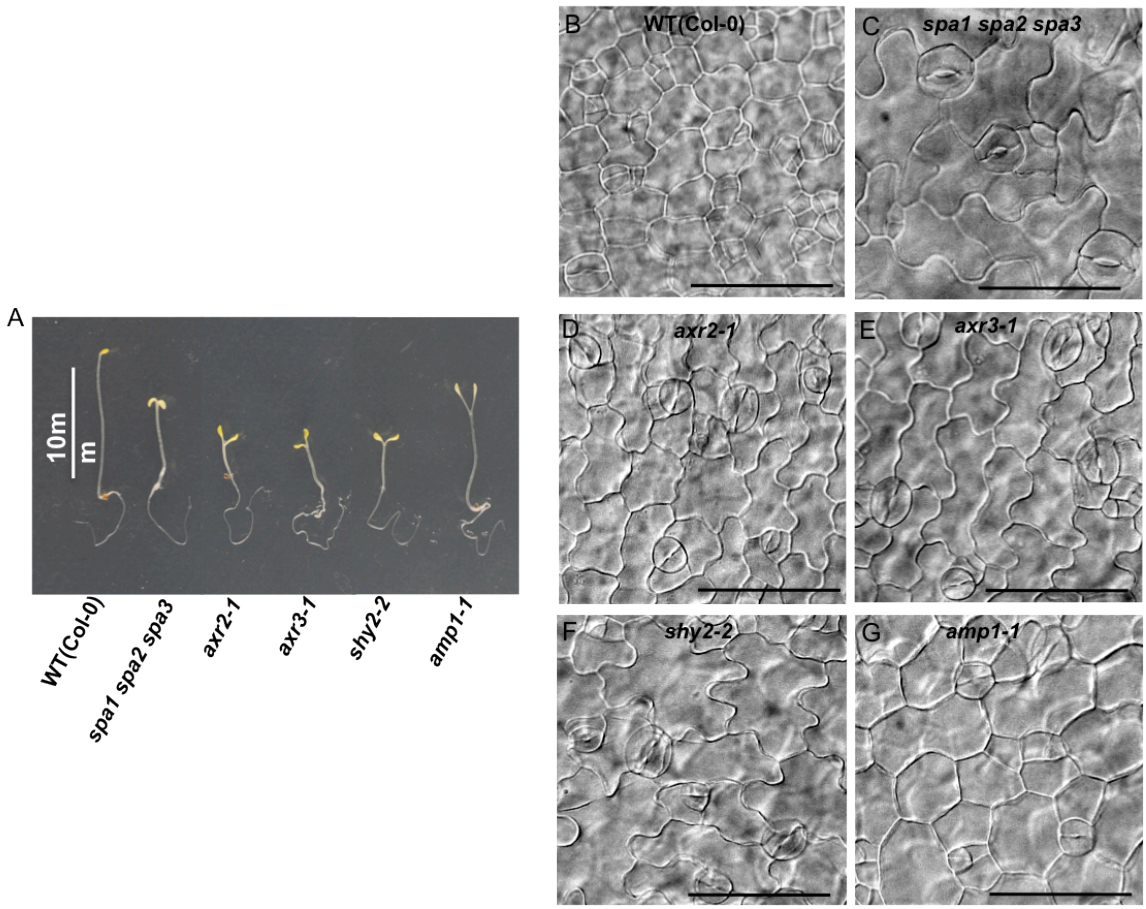

Figure 21: Phytohormone mutants show constitutive photomorphogenesis, defective stomata development and altered pavement cell shape in darkness.

(A) Visual phenotype of 4-day-old wild-type, *spa1 spa2 spa3*, *axr2-1*, *ax3-1*, *shy2-2* and *amp1-1* mutant seedlings grown in darkness.
(B) – **(G)** Abaxial cotyledon epidermis of 10-day-old dark-grown wild-type (B), *spa1 spa2 spa3* (C), *axr2-1* (D), *axr3-1* (E), *shy2-2* (F) and *amp1-1* (G) mutant seedlings. Scale bars = 50 μm

III. 2. Functional conservation of COP1 and SPA proteins across plant species

Light responses are well characterized in different plant species (Kendrick and Kronenberg; 1994). However, little is known about light signaling in plant species other than Arabidopsis. All plant species from algae and moss to angiosperms contain light sensing photoreceptors and their functions are known to a large extent (Lariguet et al., 2005; Moeglich et al., 2010), but downstream of photoreceptors, light signaling intermediates are largely uncharacterized in other plant species. The functions of *COP1* and *SPA* genes are known in detail in Arabidopsis, which is a dicotyledonous plant (Laubinger et al., 2004; Hoecker, 2005; Laubinger et al., 2006). Phylogenetic studies showed the presence *COP1* and *SPA* homologs even very early in plant evolution such as in the non-flowering moss *Physcomitrella patens* (Richardt et al., 2007). A *COP1* gene has also been identified from monocotyledonous rice (Tsuge et al., 2001), however, there is no information about *SPA* genes in rice. Here, I further identified *COP1* and *SPA* homologs in rice and Physcomitrella and then examined the functionality of the identified homologs in Arabidopsis.

III. 2. 1. Identification of Rice and Physcomitrella *COP1* and *SPA* homologs

In order to identify Rice and Physcomitrella *COP1* and *SPA* homologs, database searches were performed using full length Arabidopsis COP1 (AtCOP1) and Arabidopsis SPA (AtSPA*)* protein sequences. The databases searched were http://cdna01.dna.affrc.go.jp/ for rice and http://www.cosmoss.org/ for *Physcomitrella patens*. The database search for the AtCOP1 sequence in the rice database retrieved three cDNA sequences with accession numbers AK111614, AK112098 and AK102740, which encode proteins with 685, 604 and 356 amino acids, respectively. However, all the three retrieved cDNA sequences share the same locus ID, suggesting that the smaller cDNA sequences, AK112098 and AK102740, are truncated versions of AK111614. Moreover, with 685 amino acids, the protein product of AK111614 is similar in length to AtCOP1, which contains 675 amino acids. Therefore, I considered AK111614 as single Rice *COP1* homolog and named it as *OsCOP1*. OsCOP1 and AtCOP1 share 68% identical amino acids (Supplemental figure S14).

The Physcomitrella database search with the AtCOP1 protein sequence retrieved nine *COP1* homologs. This was consistent with the earlier phylogenetic analysis suggesting the presence of nine *COP1* homologs in Physcomitrella (Richardt et al., 2007). However, the nine COP1 proteins of Physcomitrella are highly similar to each other (Supplemental figure S13). The Physcomitrella COP1 homolog denoted as the

Phypa_167057 T21L14.11; COP1 regulatory protein [Arabidopsis thaliana] in the Physcomitrella database, showing highest blast score to AtCOP1 sequence, was named PpCOP1 and used for further functional study. PpCOP1 consists of 670 amino acids and shares 62% identical residues with AtCOP1 (Supplemental figure S14). Moreover, PpCOP1 shares 56% to as much as 84% identities to other eight COP1 proteins of Physcomitrella (Supplemental figure S13)

The rice database search for the AtSPA-like sequences retrieved three cDNA sequences with accession numbers AK111749, AK120171 and AK101974. However, AK111749 and AK120171 share the same locus ID, suggesting that AK120171 is a truncated version of AK111749. Thus, AK111749 and AK101974 were considered as two *SPA* homologs in rice. AK111749 encodes a protein with 1144 amino acids that was more similar to the AtSPA1 and AtSPA2 subclass, showing 37% and 39% identity to AtSPA1 and AtSPA2, respectively, and was, therefore, named *OsSPA1* (Supplemental figure S15). In contrast, the AK101974 encodes protein with 628 amino acids that was more similar to AtSPA3 and AtSPA4, showing 37% identity with each of these proteins (Supplemental figure S16). Therefore, it was named *OsSPA4*.

A blast search of the Physcomitrella database with the AtSPA sequences retrieved two *SPA* homologs, which are denoted as Phypa_178433 spa4 (spa1-related 4) signal transducer and Phypa_126406 spa4 (spa1-related 4) signal transducer in the database. This was striking as earlier phyolgenetic studies suggested the presence of only one SPA homolog in Physcomitrella (Richardt et al., 2007). The Phypa_178433 and Phypa_126406 were named *PpSPAa* and *PpSPAb*, respectively. *PpSPAa* and *PpSPAb* encode proteins with 804 amino acids and 756 amino acids, respectively, and are highly similar to each other in having 85% identical residues. However, I was able to amplify only one of these two *SPA* homologs (*PpSPAa*) from Physcomitrella cDNA, and hence *PpSPAa* was used for further functional studies. This PpSPAa homolog shares ~ 32% sequence identity with AtSPA1 and AtSPA2 and ~ 44% identity with AtSPA3 and AtSPA4 (Supplemental figure S15, S16).

Figure 22 illustrates the phylogenetic relationship among the COP1 and SPA proteins from Arabidopsis, Rice and Physcomitrella. The COP1 proteins from three species showed higher sequence similarity then the SPA proteins. The three COP1 proteins share 55% identical amino acid residues, whereas the SPA proteins from the three species are more divergent. Interestingly, COP1 and SPA homologs from the three species show a higher degree of conservation at the C-terminus, but exhibit very low sequence similarity at the N-terminus (Supplemental figure S14, S15, S16).

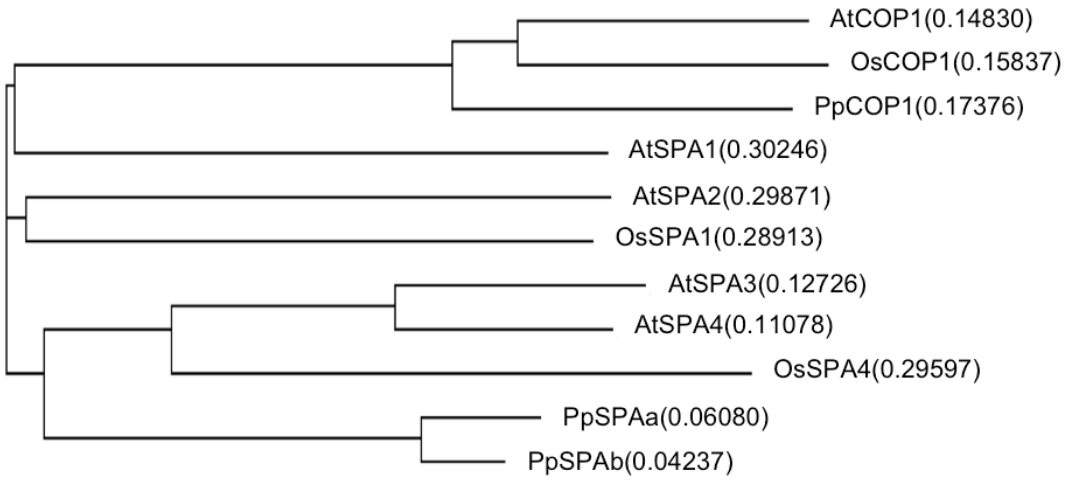

Figure 22: Phylogenetic tree showing the relationship among the COP1 and SPA proteins of Arabidopsis, rice and Physcomitrella.

The relationship is based upon comparison of full-length protein sequences using the CLUSTAL W program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Amino acid sequences of all proteins are obtained from the respective databases. The branch length is proportional to the sequence divergence. Values in brackets display tree graph distances.

III. 2. 2. Rice and Physcomitrella *COP1* are functional in Arabidopsis

In order to investigate the functionality of rice and Physcomitrella *COP1* in Arabidopsis, *OsCOP1* and *PpCOP1* were overexpressed under the control of the 35S promoter in the *cop1-4* mutant background (Figure 23). As a control, *AtCOP1* was also

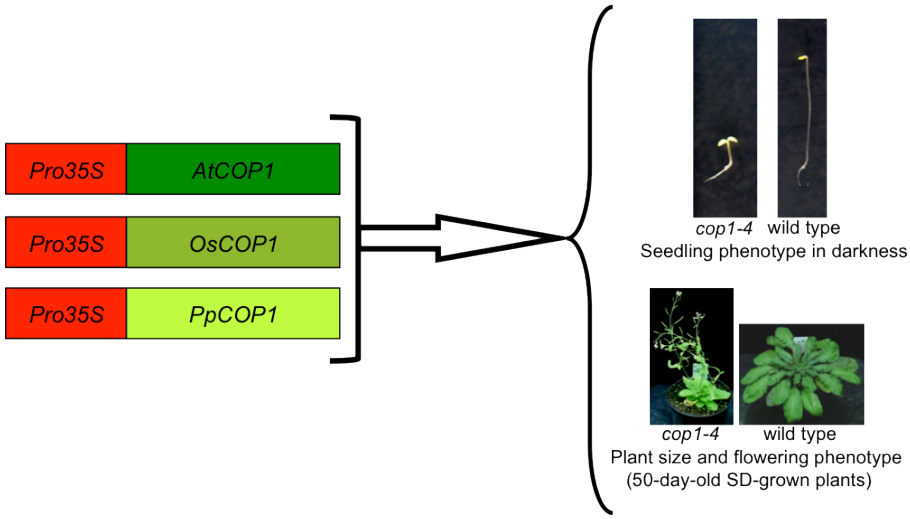

Figure 23: Strategy to study the functionality of rice and Physcomitrella *COP1* in Arabidopsis.

Rice *COP1* (*OsCOP1*) and Physcomitrella *COP1* (*PpCOP1*) were expressed under the control of the *35S* promoter in the *cop1-4* mutant. As a control, Arabidopsis *COP1* (*AtCOP1*) was also expressed under the *35S* promoter in the same mutant. Transgenic *cop1-4* mutants were analyzed for complementation of seedling, leaf size and flowering time phenotypes.

expressed under the 35S promoter in the same mutant. At least 25 independent transgenic plants were selected for each of the *35S:AtCOP1*, *35S:OsCOP1* and *35S:PpCOP1* constructs.

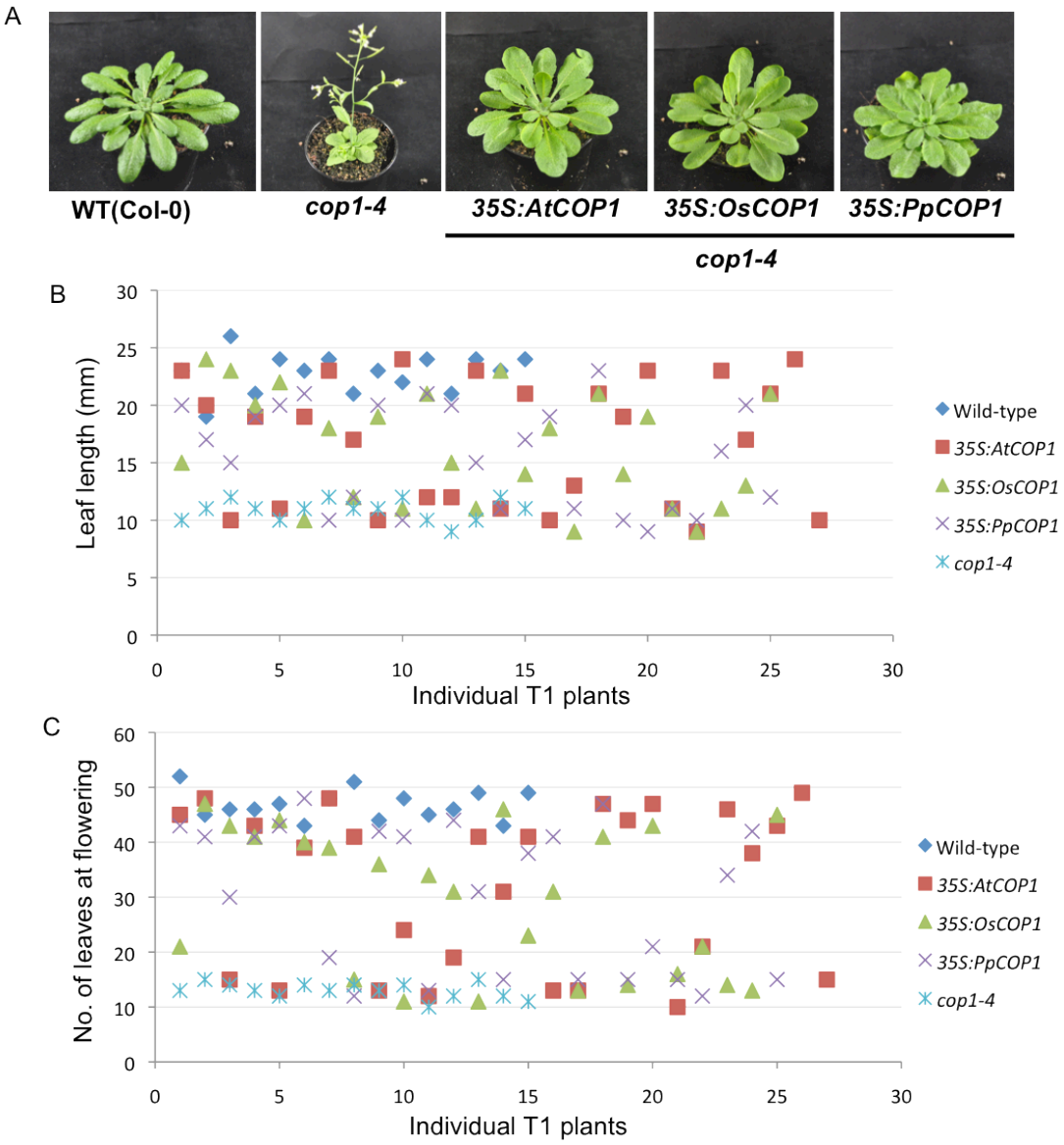

Figure 24: Rice and Physcomitrella *COP1* complement the plant size and the flowering time phenotype of the Arabidopsis *cop1-4* mutant.

(A) Visual phenotype of transgenic (T1) *cop1-4* plants expressing *35S:AtCOP1/OsCOP1/PpCOP1*.Plants were grown in short day for 45 days. Shown here are one representative T1 plant for each transgene along with wild-type (WT) and *cop1-4* mutant plants.
(B) Quantification of leaf length of the genotypes shown in (A) in T1. Scattered-plot of leaf length of at least 25 independent T1 plants for each transgene and 15 plants for controls, the wild-type and the *cop1-4* mutant, are shown. The length of the biggest leaf of 4-week-old short-day-grown plants was measured.
(C) Scattered-plot of flowering time of the short-day-grown T1 transgenic plants used in (B). Flowering time was determined by counting the number of rosette leaves at flowering.

Complementation analysis of adult plant traits, i.e. plant size and flowering time, was conducted in the T1 generation. The *cop1-4* mutant shows a smaller adult plant and leaf size as well as an early-flowering phenotype in short days when compared to the wild type (Figure 23). As expected, the *35S:AtCOP1* construct fully complemented the leaf size and flowering time phenotypes of the *cop1-4* mutant and these transgenic plants appeared similar to wild-type plants (Figure 24A). Both the *35S:OsCOP1* and the *35S:PpCOP1* constructs also led to full complementation of the leaf size and flowering time phenotypes of the *cop1-4* mutant (Figure 24A). Figures 24B and 24C show scattered-plots of the quantification of leaf size and flowering time, respectively, of at least 25 independent T1 plants harboring *35S:AtCOP1/OsCOP1/PpCOP1* constructs along with wild-type and *cop1-4* plants. 15/27, 11/25 and 11/25 T1 plants carrying *35S:AtCOP1*, *35S:OsCOP1* and *35S:PpCOP1*, respectively, showed full complementation of both leaf size and flowering time phenotypes of the *cop1-4* mutant.

The seedling phenotype of transgenic *cop1-4* plants expressing *AtCOP1*, *OsCOP1* or *PpCOP1* was analyzed in the T2 generation. The *cop1-4* mutant shows constitutive photomorphogenesis in darkness in displaying reduced hypocotyl length and open cotyledons (Figure 23). Similar to the adult plant traits, the *35S:AtCOP1* construct fully complemented the seedling phenotype of the *cop1-4* mutant. Transgenic *cop1-4* seedlings expressing *35:AtCOP1* exhibited skotomorphogenesis in displaying elongated hypocotyls and closed cotyledons in darkness (Figure 25). Transgenic *cop1-4* seedlings expressing *35S:OsCOP1* also led to full complementation of the seedling phenotype of the parental mutant. Interestingly, transgenic lines expressing *35S:PpCOP1* fully complemented the hypocotyl length phenotype, but did not complement the cotyledon phenotype (Figure 25). Altogether, these observations suggest that rice and Physcomitrella *COP1* are functional in Arabidopsis. However, Physcomitrella *COP1* showed only partial complementation at the seedling stage.

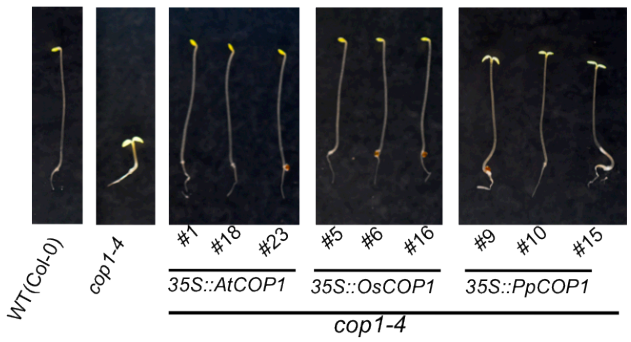

Figure 25: Rice and Physcomitrella *COP1* complements the seedling phenotype of the Arabidopsis *cop1-4* mutant.

Visual phenotype of transgenic (T2) *cop1-4* seedlings expressing *35S:AtCOP1/OsCOP1/ PpCOP1*. Four-day-old dark-grown seedlings of three independent transgenic lines for each transgene, along with the wild-type (WT) and the *cop1-4* seedling, are shown.

III. 2. 3. Rice and Physcomitrella *SPA*s are not functional in Arabidopsis

In Arabidopsis, the *SPA* genes regulate seedling development, leaf size and photoperiodic flowering with redundant and specific functions (Laubinger et al., 2004; 2006). The *spa1 spa3 spa4* triple mutant shows reduced hypocotyl length in lower fluences of R and FR, tiny-plant size and early-flowering in short days. Therefore, in order to examine the functionality of Rice and Physcomitrella SPA proteins in Arabidopsis, these were expressed under the native *SPA1* and *SPA4* promoters in the Arabidopsis *spa1 spa3 spa4* triple mutant and the resulting transgenic plants were analyzed for complementation of *spa* mutant phenotypes. *OsSPA1* and *PpSPAa* were expressed under the control of the *SPA1* native promoter and *OsSPA4* and *PpSPAa* were expressed under the control of the *SPA4* native promoter in the triple mutant. As controls *AtSPA1* and *AtSPA4* were expressed under the control of the native *SPA1* and *SPA4* promoters, respectively, in the same triple mutant (Figure 26). At least 25 independent transgenic lines were selected for each of the constructs.

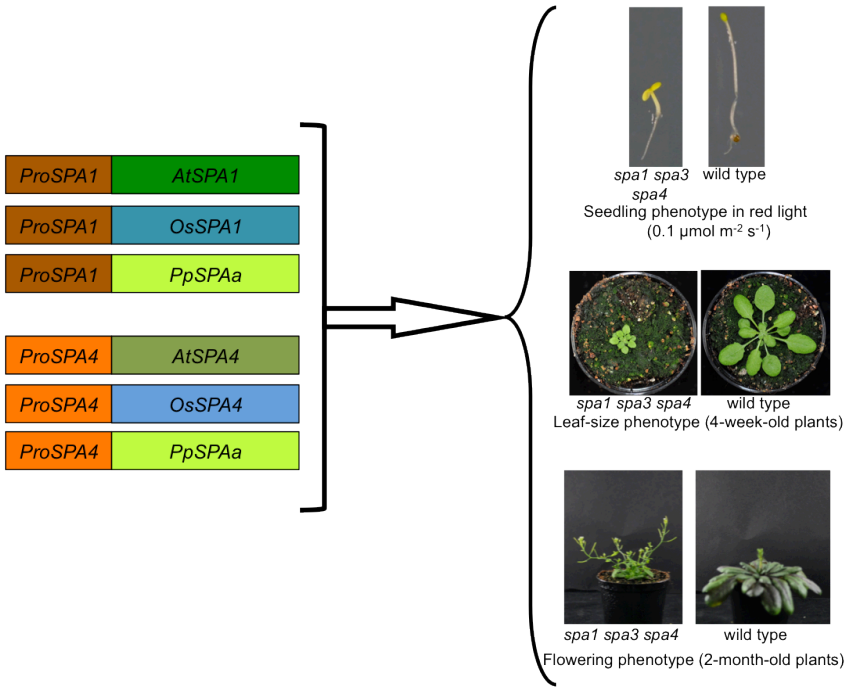

Figure 26: Strategy to study the functionality of rice and Physcomitrella *SPAs* in Arabidopsis.

OsSPA1 and *PpSPAa* were expressed under the control the native *SPA1* promoter and *OsSPA4* and *PpSPAa* under the control of the native *SPA4* promoter in the *spa1 spa3 spa4* mutant. As a control, *AtSPA1* and *AtSPA4* were also expressed under the *SPA1* and *SPA4* promoter, respectively, in the same mutant. Transgenic *spa1 spa3 spa4* mutants were analyzed for the complementation of seedling, leaf size and flowering time phenotypes.

The complementation analysis of the leaf size and flowering-time phenotype was performed in the T1 generation. *AtSPA1* is the predominant regulator of photoperiodic flowering with a significant contribution to leaf size regulation. *AtSPA4* is the

predominant regulator of adult plant and leaf size, with a minor contribution to flowering time regulation. Consistent with this, transgenic plants expressing *SPA1:AtSPA1* fully

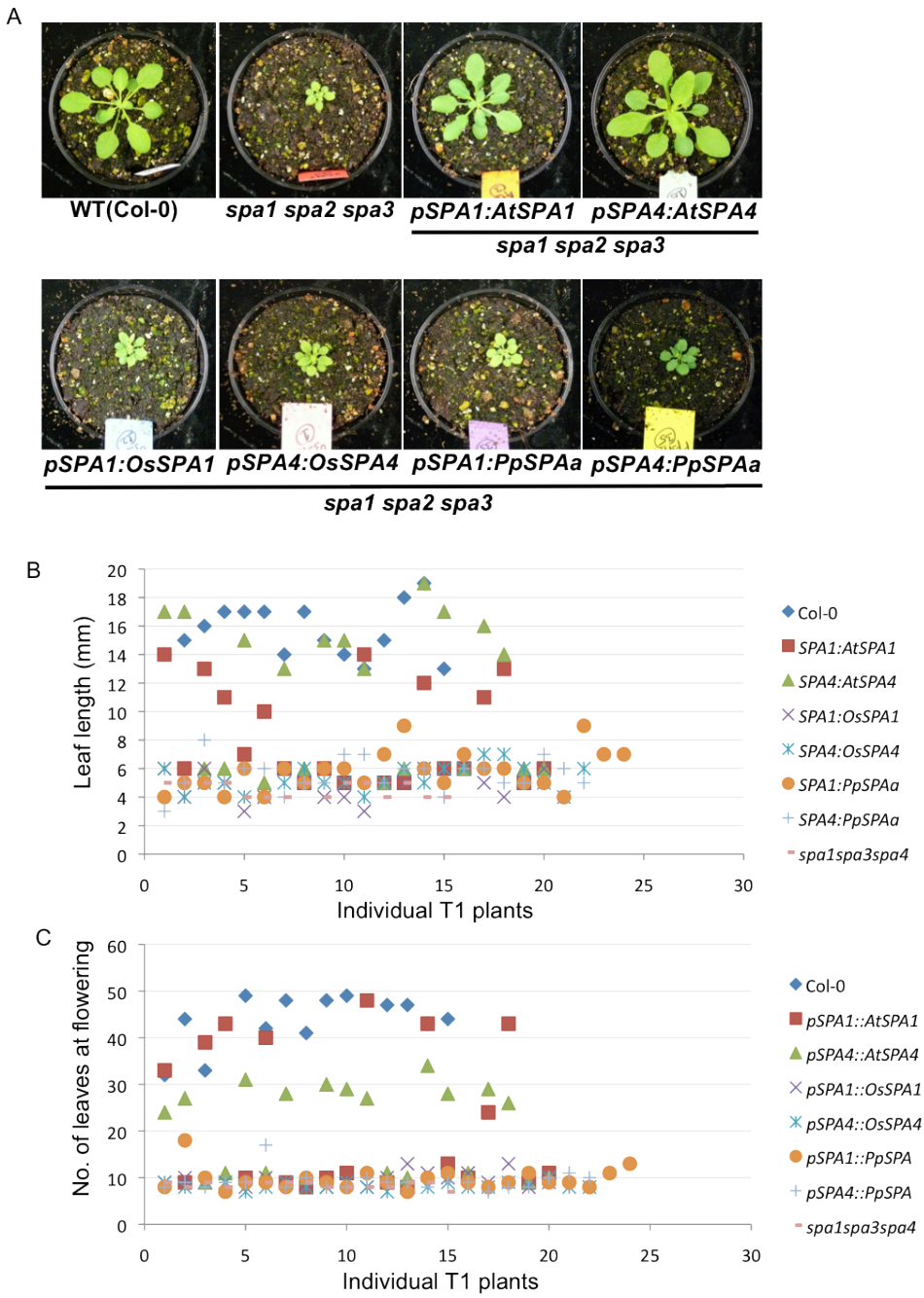

Figure 27: Rice and Physcomitrella *SPA*s do not complement the plant size and the flowering time phenotype of the Arabidopsis *spa1 spa3 spa4* mutant.

(A) Visual phenotype of transgenic (T1) *spa1 spa3 spa4* plants expressing *SPA1:AtSPA1/OsSPA1/PpSPAa* and *SPA4:AtSPA4/OsSPA4/PpSPAa* constructs. Plants were grown in short-day for four weeks. Shown are one representative T1 plant for each transgene along with controls, the wild-type (WT) and the *spa1 spa3 spa4* mutant.
(B) Quantification of leaf length of genotypes shown in (A) in the T1 generation. Scattered-plot of leaf length of at least 20 independent T1 plants for each transgene and 15 plants for controls, the wild-type (WT) and the *spa1 spa3 spa4* mutant, are shown.
(C) Scattered-plot of the flowering time phenotype of the short-day-grown T1 transgenic plants used in (B).

complemented the early-flowering phenotype and partially complemented the tiny-leaf size phenotype of the *spa1 spa3 spa4* mutant (Figure 27A, B, C). In contrast, transgenic lines expressing *SPA4:AtSPA4* fully complemented the leaf size phenotype and partially complemented the flowering time phenotype of the triple mutant. Transgenic lines expressing *OsSPA1* or *PpSPAa* under the control of the *SPA1* promoter neither complemented the leaf size nor the flowering time phenotype of the triple mutant. Similarly, transgenic plants expressing *OsSPA4* or *PpSPA*a under the control of the *SPA4* promoter also failed to complement the leaf size and the flowering time phenotype of the triple mutant (Figure 27A, B, C).

The seedling phenotype of the transgenic lines was analyzed in the T2 generation. *SPA1* is the predominant regulator of seedling development in the light, with *SPA4* also having a significant contribution. Consistent with this, the *SPA1:AtSPA1* construct fully complemented the hypocotyl length phenotype of the triple mutant under low fluences of red light and these transgenic seedlings appeared similar to wild-type seedlings (Figure 28). In contrast, the *SPA4:AtSPA4* construct partially complemented the phenotype and these transgenic seedlings had elongated hypocotyls when compared to parental *spa* triple mutant seedlings, but were still smaller than wild-type seedlings (Figure 28). Similar to the leaf size and flowering time phenotype, none of the transgenic lines expressing *SPA1:OsSPA1*, *SPA1:PpSPA*a, *SPA4:OsSPA4* or *SPA4:PpSPA* complemented the hypocotyl length phenotype of the triple mutant (Figure 28; Bachelor' thesis Laura Rupprecht, 2010). Altogether, these observations suggest that neither rice *SPA*s nor Physcomitrella *SPAa* are functional in Arabidopsis.

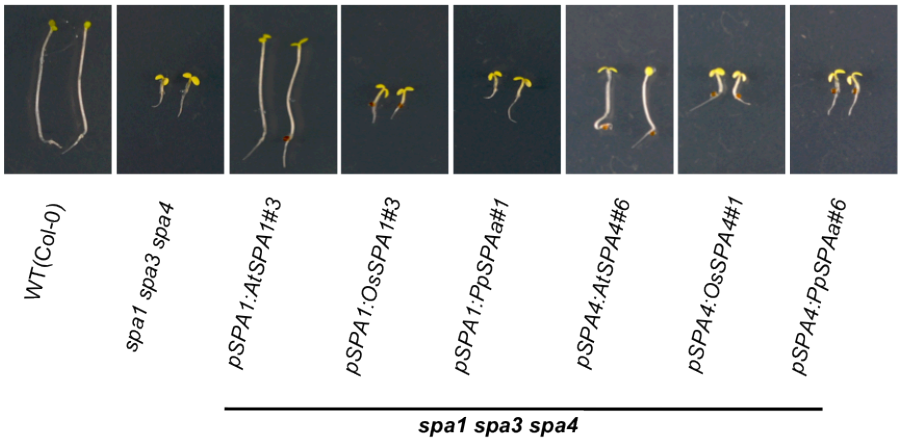

Figure 28: Rice and Physcomitrella *SPA*s do not complement the seedling phenotype of the Arabidopsis *spa1 spa3 spa4* mutant.

Visual phenotype of transgenic (T2) *spa1 spa3 spa4* seedlings expressing *SPA1:AtSPA1/OsSPA1/PpSPAa* and *SPA4:AtSPA4/OsSPA4/PpSPAa* constructs. Four-day-old dark-grown seedlings of one representative transgenic line for each transgene, along with wild-type (WT) and *spa1 spa3 spa4* seedlings are shown.

The failure of rice and Physcomitrella SPA protein-coding sequences to complement the Arabidopsis *spa* mutant phenotypes might be due to a lack of expression of the transgenes in those lines. In order to rule out this possibility, the expression of the transgenes was tested by RT-PCR, using gene-specific primers, in three representative lines for each construct. Control transgenic lines carrying *SPA1:AtSPA1* or *SPA4:AtSPA4*, which complemented all the phenotypic defects of the parental *spa* triple mutant, expressed the respective transgenes. The expression levels of these transgenic *AtSPA* genes were similar to that of the native *SPA1* or native *SPA4* gene in wild-type plants (Figure 29A, B). Transgenic plants harboring the *SPA1:OsSPA1* or *SPA1:PpSPAa*, which did not complement any of the phenotypic defects of the triple mutant, also expressed the transgenes at a significant level (Figure 29A). Similarly, *SPA4:OsSPA4* and *SPA4:PpSPAa* transgenic plants exhibited high levels of transgene expression (Figure 29B). This demonstrates that the transgenic lines containing the *SPA1:OsSPA1*, *SPA1:PpSPA*, *SPA4:OsSPA4* and *SPA4:PpSPA* in the *spa1 spa3 spa4* mutant failed to complement the mutant phenotypes in spite of expressing the transgenes.

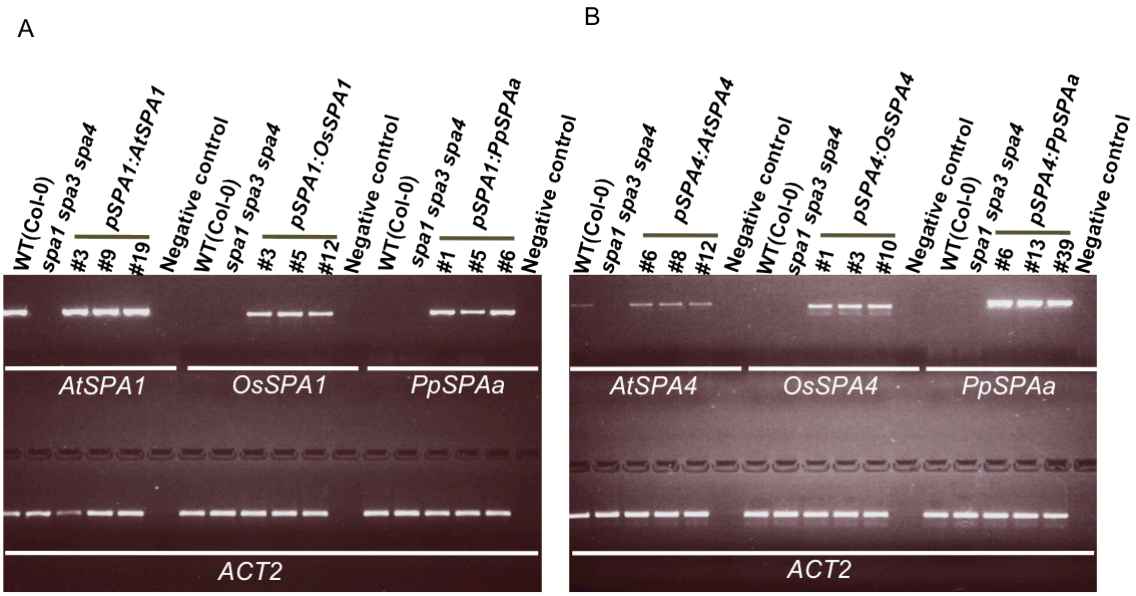

Figure 29: Transgene expression in *spa1 spa3 spa4* seedlings (T2) harboring *SPA1:AtSPA1/OsSPA1/PpSPAa* and *SPA4:AtSPA4/OsSPA4/PpSPAa* constructs.

(A) RT-PCR of *AtSPA1, OsSPA1* and *PpSPAa* (upper lanes) transcript in four-day-old seedlings. Transgene expression was checked in three independent lines for each construct. *ACT2* (lower lanes) was used as control.
(B) RT-PCR of At*SPA4, OsSPA4* and *PpSPAa* (upper lanes) transcript in four-day-old seedlings. Transgene expression was checked in three independent lines for each construct. *ACT2* (lower lanes) was used as control.

III. 2. 4. Physcomitrella SPAa physically interacts with Physcomitrella COP1 and Arabidopsis COP1

The non-functionality of rice and Physcomitrella SPAs in Arabidopsis may be due to an inability of these SPA proteins to interact with COP1. To examine this possibility, in vitro interaction studies were conducted for PpSPAa and AtCOP1 as well as PpSPAa and PpCOP1. AtSPA1 and AtCOP1 interaction was used as a positive control. Bait constructs expressing AtCOP1 or PpCOP1 under the control of the T7 promoter and prey constructs comprising the GAL4-activation domain (GAD) fused to PpSPAa or AtSPA1 were used for the synthesis of recombinant proteins by coupled transcription and translation. These proteins were then used for coimmunoprecipitation using an antibody against GAD. As reported previously, AtSPA1 interacted very well with AtCOP1 (Figure 30). Interestingly, PpSPAa also interacted with both AtCOP1 and PpCOP1, as both bait and prey were pulled down in the pellet fraction for all combinations (Figure 30). This suggests that PpSPAa can interact with AtCOP1 and that, possibly, the failure of PpSPAa to complement the Arabidopsis *spa* mutant phenotypes is not due to the failure of interaction between the PpSPAa and AtCOP1. Moreover, PpSPAa also interacts with PpCOP1 in vitro, strengthening the possibility of the formation of a COP1/SPA complex in Physcomitrella.

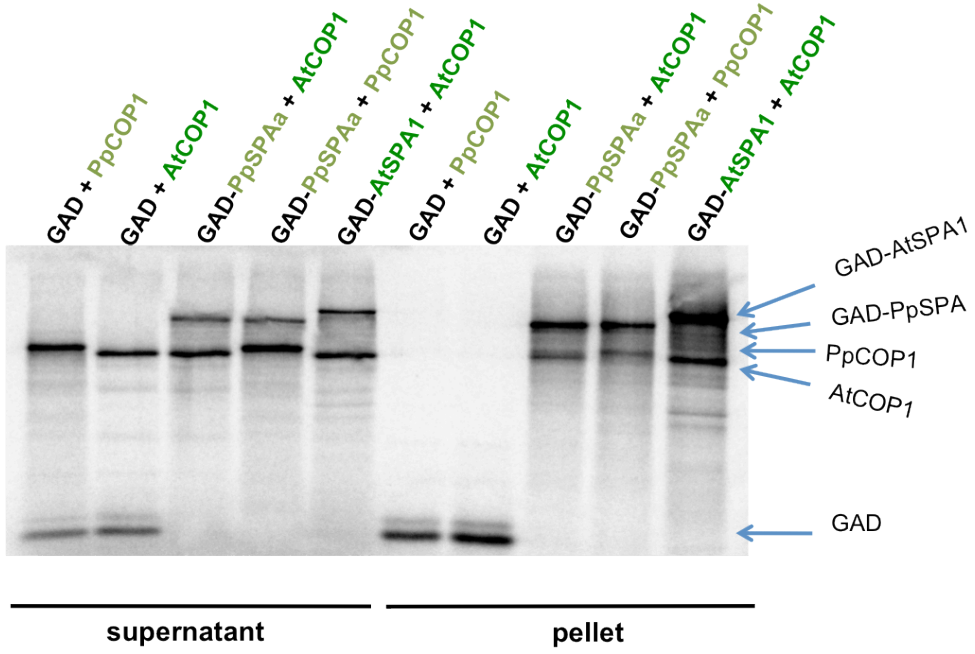

Figure 30: In vitro coimmunoprecipitation showing that PpSPAa interacts with both PpCOP1 and AtCOP1.

^{35}S-labeled full-length AtCOP1 and PpCOP1 were used as prey molecules and incubated with ^{35}S-labeled GAD-PpSPAa, GAD-AtSPA1 or GAD. Anti-GAD antibodies were used for immunoprecipitation. Supernatant and pellet fractions were resolved by SDS-PAGE and visualized by autoradiography using a phosphoimager.

IV. Discussion

IV. 1. Cell-cell communication in SPA1-regulated plant development

Plant cells cannot migrate during development, making communication over both long and short distances essential for the coordination of plant growth. This is well reflected in light-regulated plant development where light perception at one specific organ/tissue can induce responses at a distant organ/tissue. Intercellular and interorgan communications in light-regulated plant developmental processes have been observed mostly at the level of photoreceptors. Downstream of photoreceptors, SPA proteins are critical components of light signaling regulating different stages of plant development and are expressed ubiquitously in plants. In the present study, I examined the functional sites of SPA1 to regulate different stages of plant development.

IV. 1. 1. The phloem is the functional site of SPA1 to regulate photoperiodic flowering

Photoperiodic flowering in Arabidopsis involves strict spatial regulation through tissue-specific functions of photoreceptors and the CO protein (An et al., 2004; Endo et al., 2005; Endo et al., 2007). The COP1/SPA complex is critical for regulation of photoperiodic flowering in Arabidopsis by regulating CO stability through the ubiquitin – proteasome pathway (Laubinger et al., 2006; Jang et al., 2007). In the present study, I could show that SPA1 activity in the phloem fully rescued the early flowering phenotype of the parental *spa* mutant, whereas SPA1 activity in other tissues, such as the epidermis and the mesohyll, had no effect on photoperiodic flowering (Figure 11). This shows that SPA1 acts exclusively in the phloem to regulate photoperiodic flowering. Consistent with this, COP1 activity in the phloem was previously shown to regulate this process (Jang et al., 2007). These data demonstrate that the COP1/SPA complex functions in the phloem to regulate photoperiodic flowering. This is reasonable as the COP1/SPA complex has been shown to regulate CO stability via physical interaction and that the spatial expression of the CO protein is restricted to phloem companion cells (An et al., 2004; Laubinger et al., 2006; Jang et al., 2007). Moreover, CO has also been shown to act in the phloem to regulate flowering time (An et al., 2004). Taken together, COP1/SPA – CO module operates exclusively in the phloem, where it in turn regulates *FT* transcription and then the FT protein moves through the phloem as a non-cell autonomous signal to induce flowering at the shoot apex.

In Arabidopsis, cry2, phyA and phyB are major photoreceptors that regulate flowering by regulating CO stability (Guo et al., 1998; Mockler et al., 2003; Valverde et

al., 2004). cry2 promotes CO stability at the end of the day and, thus, flowering in long days (Guo et al., 1998; Valverde et al., 2004). Consistent with this, the *cry2* mutant is late flowering in long days when compared to the wild-type. The *spa1* mutant fully suppressed the late-flowering phenotype of the *cry2* mutant in both short and long days (Figure 14A; Petra Fackendahl, unpublished results). Since the same has been shown for COP1, these results demonstrate that the COP1/SPA complex functions downstream of cry2 to regulate photoperiodic flowering (Liu et al., 2008). Interestingly, cry2 has also been shown to operate in the same tissue as the COP1/SPA complex, the phloem, to regulate flowering time (Endo et al., 2005; present study). This is consistent with the suggestion that cry2 promotes CO stability by inhibiting the COP1/SPA function through physical interaction (Wang et al., 2001; Yang et al., 2001; Liu et al., 2008).

Similar to cry2, phyA is another photoreceptor promoting CO stability (Valverde et al., 2004). However, the functional site of phyA to regulate photoperiodic flowering as well the interaction of phyA with the COP1/SPA complex in this process is thus far not clear. Introduction of a *spa1* or a *cop1* mutation in the *phyA* mutant promotes flowering of the *phyA* mutant in short days as the *phyA spa1* or the *phyA cop1* mutants flower much earlier than the *phyA* mutant, although not as early as the *spa1* or the *cop1* mutants (Laubinger et al., 2006; Yu et al., 2008). This suggests that phyA, at least in part, acts by inhibiting COP1/SPA complex activity to promote CO stability. Thus, possibly, phyA may also function in the phloem to inhibit COP1/SPA complex activity and, may thereby contribute to stabilization of the CO protein, resembling cry2 function. This is further consistent with the physical interaction of phyA with COP1 (Seo et al., 2004).

phyB, in contrast to cry2 and phyA, destabilizes CO early in the day and, hence, phyB-regulated CO stability operates independently of day length. Consistent with this, the *phyB* mutant is early flowering in both long and short days (Mockler et al., 1999; Valverde et al., 2004). However, phyB-mediated CO degradation was shown to be independent of the *cop1* mutation in red light (Jang et al., 2007). Furthermore, the *phyB cop1* double mutant accumulated CO protein early in the day in contrast to the *cop1* mutant, emphasizing that phyB-regulated CO stability is independent of COP1 and, thus, the COP1/SPA complex (Jang et al., 2007). This is consistent with the finding in the present study that *spa1* and *phyB* mutations showed additive effects on flowering time in short days (Figure 14B). In the same direction, the *cop1-4 phyB-9* double mutant has also been shown to have additive effects on flowering time in short days as compared to individual single mutants (Yu et al., 2008). However, the *cop1-6 phyB* double mutant did not show additive effects and flowered very similar to the *cop1-6* under short days, which could be due to the stronger effects of the *cop1-6* allele in comparison to the *cop1-4*

(Jang et al., 2007). Interestingly, the independence of phyB- and COP1/SPA-regulated CO stability is also reflected in the terms of different functional sites of phyB and the COP1/SPA complex to regulate photoperiodic flowering. phyB has been shown to be functional in the mesophyll to regulate this process (Endo et al., 2005), whereas the COP1/SPA complex is functional in the phloem to regulate the same. Altogether, these findings demonstrate that phyB-regulated photoperiodic flowering that operates from the mesophyll is independent of COP1/SPA-regulated photoperiodic flowering that functions in the phloem and, therefore, the phyB-regulated process involves a second unknown ubiquitin ligase.

Taken together, these observations suggest that two independent pathways operating in two different spatial compartments of leaves regulate photoperiodic flowering in Arabidopsis. The module operating in the phloem involves cry2, and possibly phyA, as photoreceptors, which inhibit COP1/SPA activity in the phloem to promote CO stability in the same tissue at the end of the day under long days. CO, in turn induces *FT* expression and then FT protein moves through the phloem to induce

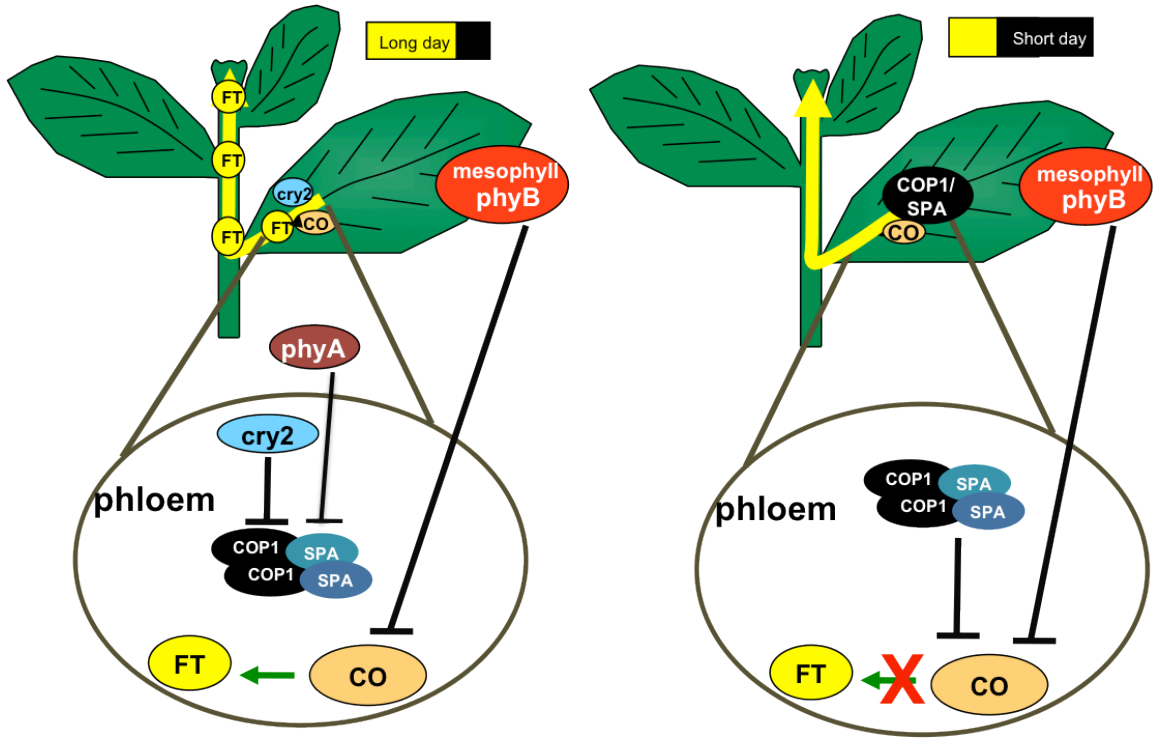

Figure 31: Spatial regulation of flowering time in Arabidopsis.

Under long days, cry2, and possibly phyA, inhibit COP1/SPA activity in the phloem to promote CO stability, which in turn induces *FT* expression. Then the FT protein moves through the phloem to induce flowering at the shoot apex. Due to COP1/SPA-mediated degradation of CO in the phloem under short days, *FT* expression is not induced, leading to delayed flowering. phyB activity in the mesophyll promotes CO degradation in the phloem, independently of the COP1/SPA complex, under both long and short days.

flowering at the shoot apex (Figure 31). In contrast under short days, CO fails to accumulate due to COP1/SPA-mediated degradation in darkness in phloem cells, leading to no *FT* transcription, and thus delayed flowering. The second mechanism, which operates from the mesophyll, involves phyB as the photoreceptor and functions independently of the COP1/SPA complex in both short and long days (Figure 31). Rather, phyB activity in the mesophyll promotes CO degradation early in the day in the phloem through an unknown ubiquitin liagse. The nature of the long distance signal coordinating CO stability in the phloem in response to phyB activity in the mesophyll is still elusive.

The COP1/SPA complex also regulates photoperiodic flowering independently of CO, since the *spa1 spa3 spa4 co* quadruple mutant showed an intermediate flowering-time phenotype as it flowered earlier than the wild type but later than the *spa1 spa3 spa4* mutant (Petra Fackendahl, unpublished results). Similarly, *cop1 co* plants also flowered earlier than wild-type plants under short days (Jang et al., 2008). However, SPA1 activity in the phloem fully complemented the early-flowering phenotype of the *spa1 spa3 spa4* triple mutant suggesting that CO-independent mechanisms regulating photoperiodic flowering are also dependent on SPA1 function in the phloem. This CO-independent regulation of photoperiodic flowering through the COP1/SPA complex may involve *CO-like* (*COL*) genes. *COL* genes do not have an obvious function in wild type plants and this may be due to COP1/SPA-mediated degradation of COL proteins as these proteins have a CCT domain through which they could be targeted by the COP1/SPA complex (Ledger et al., 2001; Griffiths et al., 2003; Robson et al., 2001; Laubinger et al., 2006; Jang et al., 2007). However, in *cop1* or *spa* mutants, these COL proteins might over-accumulate and, therefore, their function might become visible, leading to an intermediate flowering time phenotype of *cop1 co* and *spa co* mutants. Additionally, miR172-mediated photoperiodic flowering may also be a component of CO-independent mechanisms of flowering time regulation. The functional role of miR172 to mediate photoperiodic flowering depends upon GIGANTEA (GI), but is independent of CO (Jung et al., 2007). The suggested regulation of GI-stability through COP1 may indicate a possible involvement of miR172 in the regulation of photoperiodic flowering downstream of COP1/SPA complex independently of CO (Yu et al., 2008). The fact that microRNAs have been identified as a mobile signal to regulate plant developmental processes makes microRNA172 even more interesting in the context of non-cell autonomous regulation of photoperiodic flowering (Kehr and Buhtz, 2008).

Classical grafting experiments established the spatial regulation of photoperiodic flowering in other plant species also. Homologs of *CO* and *FT* have been identified in various plants species and *FT* homologs are characterized as activators of flowering in

many plant species such as rice, tomato, tobacco, *Pharbitis* and poplar (Kojima et al., 2002; Hsu et al., 2006; Lifschitz et al., 2006; Hayama et al., 2007). Interestingly, FT homologs have been identified as the systemic signal also for photoperiodic responses involved in tuberization in potato and bud set in trees such as poplar (Bohlenius et al., 2006; Rodriguez-Falcon et al., 2006). This emphasizes the broader roles of long distance signaling through FT protein in response to photoperiodic stimuli. However, tissue-specific functions of the components regulating photoperiodic responses and *FT* expression are largely unknown in other plant species. Establishing the tissue-specific functions of the various components of photoperiodic responses in other plant species in future will help to establish the long distance signaling in diverse aspects of plant development through FT protein.

IV. 1. 2. SPA1 activity in phloem cells has major effects on seedling growth

Photoreceptors have been shown to regulate hypocotyl elongation through non-cell autonomous effects as cotyledon-localized phytochromes regulate hypocotyl elongation in response to the light environment (Black and Shuttleworth, 1974, Endo et al., 2005; Warnasooriya et al., 2009). Downstream of photoreceptors, the COP1/SPA complex is a critical light signaling intermediate (Hoecker, 2005). The present study revealed that SPA1 also regulates hypocotyl elongation through non-cell autonomous effects and SPA1 activity in the phloem is the primary determinant of hypocotyl elongation in both darkness and light as it mostly complemented the hypocotyl phenotype of the *spa* mutant in darkness and partially complemented the phenotype in the light (Figure 18, 19).

Contrary to SPA1, phyB has been shown to function in the cotyledon mesophyll to regulate hypocotyl elongation. Expression of a phyB-GFP fusion in mesophyll cells of cotyledons complemented the hypocotyl-length phenotype of the *phyB* mutant (Endo et al., 2005). Moreover, inhibiting phytochrome activity in the cotyledon mesophyll led to hypocotyl elongation in red light (Warnasooriya et al., 2009). phyB activity in the phloem had no effect on hypocotyl elongation (Endo et al., 2007), and thus phyB and SPA1 act in different spatial compartments to regulate hypocotyl elongation, which is similar to the non-cell autonomous effects of phyB on the CO/FT module in the regulation of photoperiodic flowering. However, *cop1* is fully epistatic to *phyB* (Deng and Quail, 1992), suggesting phyB- and COP1/SPA-regulated seedling development are not independent of each other and phyB activity in the mesophyll might still control COP1/SPA activity in the phloem through an indirect mechanism. Additionally, the possibility that SPA1 functions redundantly in several tissues, including the mesophyll, cannot be fully excluded, since fusion of GUS to the SPA1 protein may limit SPA1 function.

Inhibiting phytochrome activity in the cotyledon mesophyll also promoted hypocotyl elongation in far-red light suggesting that phyA may also act in the mesophyll to regulate hypocotyl elongation (Warnasooriya et al., 2009). However, phyA activity in the phloem still needs to be tested as the possibility of phyA activity in the phloem besides its activity in the mesophyll can not be ruled out completely. In fact, phyA activity in the hypocotyl has been observed to effect hypocotyl elongation suggesting the importance of phyA activity outside the cotyledon mesophyll (Warnasooriya et al., 2009). Moreover, SPA1-mediated regulation of hypocotyl length in the light is dependent upon presence of phyA, raising the possibility of SPA1 and phyA acting together in the same tissue (Hoecker et al., 1998; 1999). In contrast to phytochromes, cry2 activity in mesophyll cells has no effect on hypocotyl elongation, rather it shows partial effects when acting in the phloem and the epidermis, more similar to SPA1 (Endo et al., 2007; Figure 18, 19). This suggests that cry2 and COP1/SPA may act in the same compartment to regulate hypocotyl length, as in the case of photoperiodic flowering.

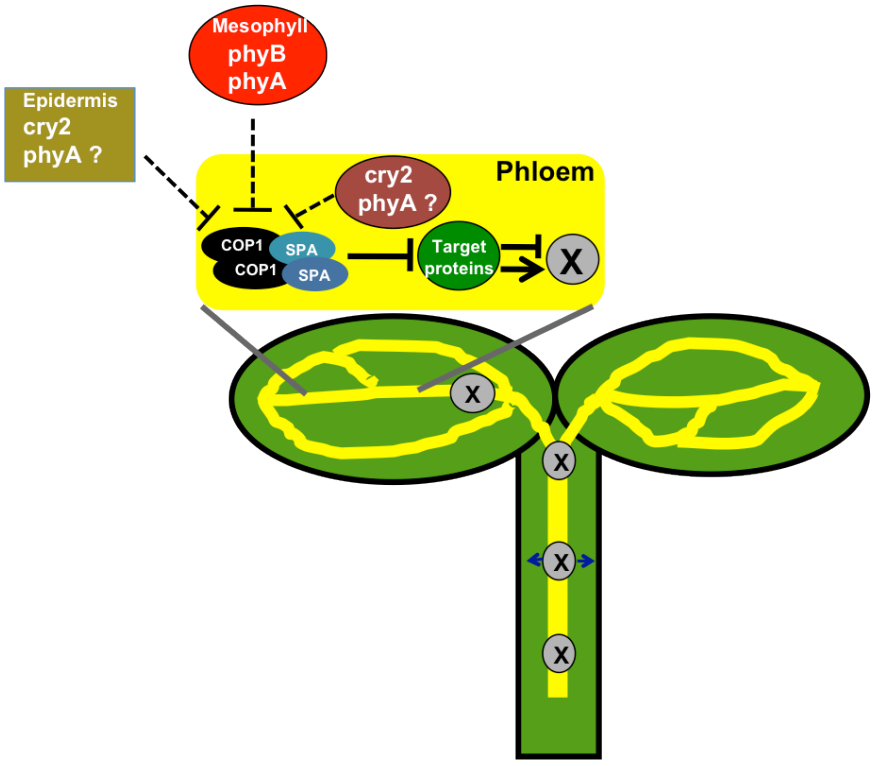

Figure 32: Spatial regulation of SPA1-controlled hypocotyl elongation.

The COP1/SPA complex activity in the phloem regulates the stability of downstream transcription factors in the same tissue, which in turn regulate hypocotyl elongation. The mobile signal X most likely operates downstream of transcription factors or other targets and may involve phytohormone or phytohormone signaling components. Active photoreceptors in mesophyll, phloem or epidermis cells may inhibit COP1/SPA function in the phloem through uncharacterized mechanisms. phyA activity in the phloem and the epidermis still needs to be tested.

Taken together, these observations suggest that in darkness, when photoreceptors are not active, COP1/SPA activity in the phloem leads to hypocotyl elongation, whereas in the light, active photoreceptors in cotyledon mesophyll (phyB and phyA), phloem and epidermis (cry2 and probably phyA) cells inhibit COP1/SPA activity in the phloem to inhibit hypocotyl elongation (Figure 32). Thus COP1/SPA activity in the phloem may serve as a connector of light perception through cotyledon-localized phytochromes and light responses in hypocotyls. This is quite reasonable as cotyledons are primary organs to perceive light signals at the seedling stage and the phloem is the structure connecting the hypocotyl to cotyledons.

Hypocotyl elongation at the seedling stage only involves longitudinal cell expansion and no cell division (Gendreau et al., 1997). Therefore, SPA1 activity in the phloem must lead to expansion of cells in outer layers. The non-cell autonomous signaling molecules regulating cell expansion in outer layers and, thereby, hypocotyl elongation in response to SPA1 activity in the phloem are not known. One possibility could be movement of the SPA1 protein itself. The restricted tissue-specific expression pattern of GUS-SPA1 in the present experiments rules out this possibility, although the observed immobility of GUS-SPA1 may also be due to GUS fusion. However, the large size of the SPA1 protein (115 kDa), which is beyond the size exclusion limit of plasmodesmata found in the sieve element-companion cell complex (27 kDa) as well as the constitutive nuclear localization of SPA1 protein and immobility of GFP-SPA1 fusion in particle bombardment of epidermal cells, further support the idea of an immobile SPA1 protein (Hoecker et al., 1999; Imalu et al., 1999).

Thus, the long distance signaling molecules likely operate downstream of the SPA1 proteins. The next possibility could be the movement of proteins targeted by the COP1/SPA complex, such as the transcription factors HY5, HFR1 and HYH, to regulate cell elongation in outer layers. This could well be possible for HY5 and HYH, which are only 18.4 kDa and 15.2 kDa, respectively in size, and therefore far below the size exclusion limit of the sieve element-companion cell complex (Imalu et al., 1999). However, due to their constitutive nuclear localization as well as immobility in particle bombardment of epidermal cells, cell-to-cell movement of these transcription factors is highly unlikely (Ang et al., 1998; Chattopadhyay et al., 1998; Fairchild et al., 2000). These observations suggest that transcription factors, downstream of the COP1/SPA complex, also probably function in the phloem to regulate hypocotyl elongation. Thus, it is likely that the communicating signal either operates downstream of these transcription factors or involves other unknown targets of the COP1/SPA complex.

Phytohormones are likely to be involved in hypocotyl elongation downstream of COP1/SPA-targeted transcription factors. Auxin is of particular interest among the

phytohormones as it has been shown to be involved in the regulation of transgene expression in the hypocotyl in response to phytochrome activity in cotyledons (Tanaka et al., 2002; Vandenbussche et al., 2005). Moreover, auxin transport is required for hypocotyl elongation in light-grown seedlings as well as elongation of petioles at the adult plant stage during the shade avoidance syndrome (Jensen et al., 1998; Tao et al., 2008). Light imposes a strong influence on multiple facets of the auxin system, controlling auxin levels, transport and responsiveness. However, polar auxin transport is not required for hypocotyl elongation in darkness, suggesting that this process, probably, depends more on auxin biosynthetic levels or on auxin responses than on auxin transport (Jensen et al., 1998). Little is known about auxin biosynthesis in the hypocotyl and, hence, determining the levels of auxin in the hypocotyls of dark-grown wild-type and *spa/cop1* mutant seedlings will be a way to address this issue.

The possible involvement of auxin signaling downstream of COP1/SPA activity in the phloem is highly likely as HY5 and HFR1 integrate light and phytohormone signaling in Arabidopsis (Cluis et al., 2004; Sessa et al., 2005). Auxin signaling is elevated in *hy5* mutant seedlings because of decreased expression of IAAs, which are negative regulators of auxin signaling (Cluis et al., 2004). IAA14, whose expression is directly regulated by HY5, acts non-cell autonomously from the vascular bundle to control lateral root development (Cluis et al., 2004; Fukaki et al., 2005). In the same direction, IAA19, which has been shown to effect growth response of the hypocotyl, is strongly expressed in the vasculature (Tatematsu et al., 2004). Further, *hfr1* mutant seedlings also show upregulation of auxin signaling pathway (Sessa et al., 2005). This suggests that, downstream of HY5 and HFR1, IAA proteins may act in a non-cell autonomous way to regulate hypocotyl elongation and the long distance signal may act even downstream of IAA proteins. If this is the case, COP1/SPA-mediated degradation of HY5 and HFR1 in dark-grown seedlings would lead to elevated auxin signaling, which in turn leads to hypocotyl elongation. Consistent with this, *iaa* gain-of-function auxin resistant mutants, having reduced or inhibited auxin signaling, show constitutive photomorphogenic phenotype in darkness (Nagpal et al., 2000; Tian et al., 2002). In contrast, HY5 and HFR1 are active in light that lead to transcription of negative regulators of auxin signaling leading to reduced auxin signaling and, thus inhibiting hypocotyl length.

Besides auxin, cytokinin may also constitute a component of long distance signaling downstream of the COP1/SPA complex. Elevated cytokinin doses can mimic the *cop1* phenotype in darkness (Chory et al., 1994). Moreover, *cop1-4* mutants showed strongly elevated expression of a cytokinin-responsive gene, ARR6, in darkness (data not shown). This could be due to increased cytokinin levels in *cop1/spa* mutants in

darkness. The COP1/SPA complex may link to cytokinin signaling through HFR1 as the *hfr1* mutant shows elevated expression of CKX5, a cytokinin-degrading enzyme (Sessa et al., 2005). Thus, in darkness, due to COP1/SPA mediated degradation of HFR1, CKX5 is expressed at higher levels leading to a reduced cytokinin content, which in turn may contribute to hypocotyl elongation. This hypothesis can be tested by quantifying the cytokinin levels in *cop1/spa* mutants in comparison to the wild-type. Besides auxin and cytokinin, other phytohormones such as gibberellic acid, ethylene and brassinosteroids (BR) are also involved in regulation of hypocotyl length and, hence, these phytohormones or their downstream signaling components may also be involved in cell-cell communication to regulate hypocotyl length (Vandenbussche et al., 2005). Consistent with this, BR activity in the epidermis regulates hypocotyl length through non-cell autonomous effects on cell expansion (Savaldi-Goldstein et al., 2007).

Apart from diffusible substances, one possibility to explain the SPA1-regulated hypocotyl elongation through its activity in the phloem could be that mechanical stimuli from the inner vasculature act on outer cells. COP1/SPA activity in the phloem cells could induce elongation of phloem cells, the most rigid tissue of the seedling architecture, which in turn could lead to active or passive expansion of cells in outer layers due to mechanical pressure from inner cells, thus leading to hypocotyl elongation. This is supported by the previous observation that local mechanical wall relaxation can induce growth of the surrounding cells via cell division-independent mechanisms (Fleming et al., 1997; Pien et al., 2001).

IV. 1. 3. SPA1 activity in both the phloem and the mesophyll contribute to leaf size

Adult *spa* quadruple as well as *cop1-4* plants are extremely tiny, emphasizing the role of the COP1/SPA complex in normal adult plant growth (Schwechheimer and Deng, 2000; Laubinger et al., 2004). The present study revealed that SPA1 imparts non-cell autonomous effects from both phloem and mesophyll cells to regulate plant and leaf size (Figure 12). However, neither SPA1 activity in the phloem nor in the mesophyll fully complemented the tiny leaf size of the parental *spa* mutant. Combining the SPA1 activity in both phloem and mesophyll cells together led to full complementation of the leaf size phenotype of the parental *spa* mutant, indicating that SPA1 activity in both the mesophyll and the phloem is important for regulation of this process (Figure 13). Brassinosteroids (BR), which are important growth-promoting phytohormones, affect leaf size non-cell autonomously through its activity in the epidermis, whereas signals from the vasculature do not contribute to this regulation (Savaldi-Goldstein et al., 2007). In contrast, SPA1

does not function in the epidermis to regulate leaf size. This suggests that BR-mediated leaf size regulation is likely independent of light-mediated leaf size regulation through the COP1/SPA complex (Figure 33). In addition ANGUSTIFOLIA (AN), an important component of leaf shape regulation, has been shown to function in both epidermis and sub-epidermis to regulate leaf length and in sub-epidermis to regulate leaf width (Bai et al., 2010). Together, these observations emphasize the complexity of leaf size and leaf shape regulation, which involves all three tissue layers of the leaf. Recent observations that multiple pathways involving different phytohormone signaling independently converge on leaf size control in Arabidopsis further support this idea (Gonzalez et al., 2010).

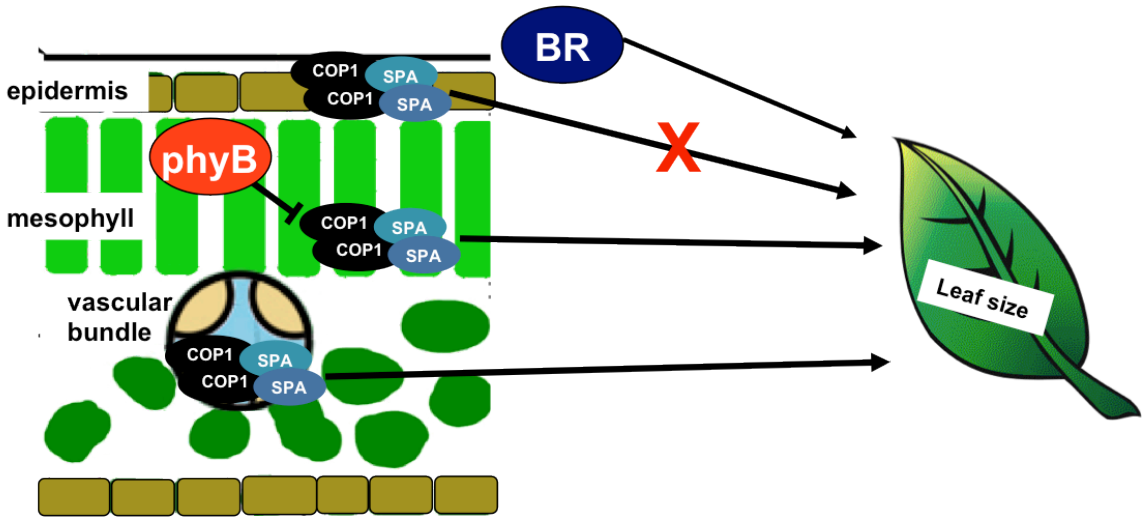

Figure 33: Spatial regulation of SPA1-dependent leaf size control.

SPA1 activity in both the phloem and the mesophyll is important for leaf size regulation. Thus SPA1 activity is likely independent of BR-regulated leaf size, operating from the epidermis. The COP1/SPA complex acts downstream of phyB in the mesophyll to regulate leaf size.

Among the photoreceptors, phyB is required for normal leaf development and the *phyB* mutant has smaller leaf blades when compared to the wild type. Based on an enhancer trap study, phyB has been suggested to act in mesophyll cells to regulate leaf size (Endo et al., 2005), suggesting that SPA1 and phyB may act in the same pathway to regulate leaf size from the mesophyll. Consistent with this, the *spa1* mutation fully suppressed the leaf size phenotype of the *phyB* mutant, indicating that *SPA1* acts downstream of *PHYB* in this pathway (Figure 14C). Moreover, the *cop1-6 phyB-9* double mutant also showed smaller leaves, similar to the *cop1-6*, when compared to the *phyB-9* mutant, suggesting that the COP1/SPA complex may act downstream of phyB in mesophyll cells to regulate leaf size (Petra Fackendahl, unpublished results) (Figure 33). However, phyB activity in the phloem had no effect on the leaf size (Endo et al., 2005),

whereas, SPA1 activity in the phloem contributes to leaf size regulation, suggesting that SPA1-regulation of leaf size from the phloem could be independent of phyB, similar to the regulation of photoperiodic flowering.

The *spa1 spa3 spa4* mutant shows reduced leaf size due to reductions in both cell division and cell elongation in epidermal and mesophyll layers (Petra Fackendahl, unpublished result). Therefore, SPA1 activity in the mesophyll and the phloem needs to coordinate cell division and expansion in the other layers of leaf i.e. the epidermis. Thus in contrast to hypocotyl elongation, which only involves cell elongation, leaf size control is dependent on both cell elongation and division.

One explanation for this could be that physical forces generated by SPA1 activity in the phloem or mesophyll cells may trigger cell division and expansion in other layers. The existence of such a mechanism is suggested by the finding that exogenous application of expansin on the shoot apical meristem could trigger local outgrowth (Fleming et al., 1997; Pien et al., 2001). The second possibility could be that the SPA1 activity in the phloem or the mesophyll promotes cell division in other cell layers via non-mechanical processes. Phytohormones or phytohormone-signaling components may constitute the mobile signal regulating cell division downstream to the COP1/SPA complex, as discussed later. However, the COP1/SPA complex may also directly modulate the cell cycle as COP1 has been shown to regulate levels of E2F transcription factors, which play an important role in cell cycle regulation, during meristem activation (Lopez-Juez et al., 2008). Interestingly, components of the cell cycle regulatory machinery like the cyclin-dependent kinase inhibitor ICK1/KRP1 has been shown to act non-cell autonomously through its movement between cells (Weinl et al., 2005), suggesting that COP1/SPA activity in specific tissues might regulate cell division in other layers through diffusible components of the cell cycle.

The cell-to-cell movement of proteins is considered to be crucial in plant development. For example, the homeobox protein KNOTTED1 (KN1) controls leaf formation through its movement from inner cells to epidermal cells (Lucas et al., 1995; Kim et al., 2002). SPA-regulated leaf size may also involve cell-cell movement of proteins. Known substrates of the COP1/SPA complex appear to have no or little function in the control of plant size. Though, expressing CO from a phloem-specific promoter has been shown to reduce the plant size (An et al., 2004), the small leaf phenotype of *spa1 spa3 spa4* was independent of *CO* (Petra Fackendahl, unpublished results), suggesting that long distance transport of FT, downstream of CO, has no role in SPA-dependent leaf size regulation. Similarly, the *hfr1* mutation also did not affect the leaf size of the *spa1 spa3 spa4* triple mutant. HY5 is, at least, partially involved in the COP1/SPA-dependent leaf size regulation as the *hy5 spa1 spa3 spa4* quadruple mutant

and the *hy5 cop1-4* double mutant showed slightly larger leaves than the *spa1 spa3 spa4* and the *cop1-4* mutant, respectively (Petra Fackendhal, unpublished results). However, cell-to cell movement of HY5 is highly unlikely as it is constitutively localized in the nucleus (Chattopadhyay et al., 1998). Overall these results suggest that COP1/SPA-dependent leaf size regulation mostly involves so far unknown targets.

As discussed for SPA1 signaling in seedlings, phytohormones or components of phytohormone signaling also represent potential candidates for diffusible signals to regulate leaf size in response to the COP1/SPA complex. Phytochrome regulation of leaf morphology is partly the result of changes in auxin levels (Morelli and Ruberti, 2000). In the same direction, various auxin-responsive genes are upregulated in leaves in response to shade, which in turn arrest growth of the leaf blade (Kozuka et al., 2010). Thus, non-cell autonomous effects downstream to the COP1/SPA complex may involve changes in auxin levels or auxin signaling. Potentially, COP1/SPA complex may modulate auxin signaling through involvement of downstream target transcription factors.

Interestingly, reduced expression of the cytokinin responsive gene *ARR6* in the *cop1-4* mutant leaves (data not shown), suggests that the reduced plant size of *spa* and *cop1* mutants may also be a result of reduced cytokinin levels and/or signaling in these mutants, which in turn may lead to reduced cell division. Various genes involved in cytokinin biosynthesis and degradation such as the cytokinin-biosynthetic gene *IPT3* and the cytokinin-degradation gene *CKX6* as well as various *ARABIDOPSIS RESPONSE REGULATORS* (*ARR*s) show vascular bundle-specific expression, pointing towards cytokinin or its signaling components being one of the diffusible components involved in SPA1-regulated leaf development from the phloem (Werner et al., 2003; Miyawaki et al., 2004; Hirose et al., 2008). Interestingly, reduced leaf growth in the shade avoidance syndrome, and thus in the *phyB* mutant, involves auxin-mediated cytokinin degradation in developing vasculature (Carabelli et al., 2007). Since SPA1 is required for reduced leaf blade growth of the *phyB* mutant (Figure 14C), the COP1/SPA complex may link phyB and the downstream modulators of phytohormone signaling to affect leaf size. Comparing the auxin and cytokinin levels in the leaves of wild-type and *spa* mutants will be the first step to link the SPA-regulated leaf size control to phytohormone signaling. Tissue-specific expression of phytohormone signaling components in *spa* mutant plants will, further, add up to understanding the involvement of phytohormone or its signaling components as diffusible signal to regulate leaf size.

Mature leaves of Arabidopsis detect and transmit the external environmental information to new leaves of the same plant. This has been shown for stomata development in response to light and CO_2 where long distance signaling from matured leaves regulates stomata density of new leaves (Lake et al., 2001). Since the phloem is

the primary structure connecting the older leaves to new leaves, it is tempting to assume the involvement of the phloem in such long distance signaling between these organs. Consistent with this, SPA1 activity in the phloem may also serve as a way of communication between the developed leaves and young developing leaves in order to determine the leaf size. This is further supported by the observation in the present study that phloem-specific expression of GUS-SPA1 under the *SUC2* promoter showed strong expression in matured leaves, but lower expression in young emerging leaves. Generating chimeric transgenic plants expressing SPA1 exclusively in fully developed leaves, without any expression in young emerging leaves, can test this hypothesis.

IV. 1. 4. Phloem-specific SPA1 activity regulates stomata differentiation and pavement cell shape in darkness

Recently, COP1 and SPA proteins have been identified as key negative regulators of stomata differentiation (Kang et al., 2009). The *spa1 spa2 spa3* triple mutant shows constitutive stomata differentiation in darkness. SPA1 expression under the control of the native *SPA1* promoter fully complemented the stomata phenotype of the triple mutant suggesting SPA1 is a major regulator of stomata development in darkness, similar to its role in de-etiolation. Interestingly, SPA1 activity in the phloem, but not in any of the other tissues tested, fully rescued the stomata phenotype of the parental mutant (Figure 20), suggesting that SPA1 acts exclusively in the phloem to regulate stomata differentiation. The finding of a non-cell autonomous signal from the vasculature regulating stomata development is quite intriguing. Recently, *STOMAGEN*, which is a positive regulator of stomata development, has been shown to be functional in the mesophyll to regulate stomata differentiation (Sugano et al., 2010). These observations suggest that non-cell autonomous signals from the inner tissue layers play an important role in stomata differentiation in the epidermal layer.

The nature of the long-distance signals from the phloem to regulate stomata differentiation is completely unknown. Phytohormones, regulating almost all plant developmental processes and being mobile in plants, could be the potential candidates for this. However, little is known about the roles of phytohormones in stomata formation. Gibberellins, together with auxin and ethylene, have been shown to strongly induce stomata formation in the hypocotyl (Saibo et al., 2003). Hormonal treatments specifically induce cell divisions in the epidermal layer to promote stomata development during hypocotyl growth, indicating that phytohormones or phytohormone signaling components may constitute the non-cell autonomous signal to differentiate stomata in the epidermis (Saibo et al., 2003). Consistent with this, defective stomata phenotypes of the

phytohormone mutants in the present experiments also suggest the possible involvement of phytohormones in the COP1/SPA-regulated stomata differentiation in darkness. The phenotype of *iaa* gain-of-function auxin resistant mutants showing stomata differentiation in darkness suggests that auxin may inhibit stomata development in darkness in wild-type cotyledons (Figure 21). On the other hand, a cytokinin overproducer mutant showing constitutive stomata development in darkness indicates that cytokinin may promote stomata development in those conditions. This is further consistent with the increased expression of cytokinin-responsive genes in the *cop1-4* mutant in darkness (data not shown), which shows constitutive stomata differentiation. These observations emphasize the possibility of a link of arrested stomata differentiation in darkness to phytohormones.

Interestingly, SPA1 activity in the phloem also fully complemented the lobed phenotype of the epidermal pavement cells of the parental *spa* triple mutant. However, SPA1 activity in the epidermis also had some cell-autonomous effects on the lobing of pavement cells (Figure 20). Phenotypic analysis of phytohormone mutants suggests the involvement of phytohormones, at least auxin, in this process. *iaa* gain-of-function auxin resistant mutants showed lobed epidermal cells in darkness suggesting that auxin signaling is required for the inhibition of lobing of epidermal pavement cells in darkness (Figure 21). Additionally, the lobed epidermal-cell phenotype of the *spa1 spa2 spa3* triple mutant in darkness might also be linked to defective auxin signaling in these mutants. Epidermal pavement cell shape is determined by the organization of microtubules and microfilaments and light has been shown to regulate the expression of *ACTIN7* and *TUBULIN* genes in the hypocotyl through the involvement of phytochromes (Leu et al., 1995; Mcdowell et al., 1996). Thus, non-cell autonomous effects, probably by phytohormones or phytohormone signaling components, might regulate pavement cell shape through their effect on microtubules and microfilaments in response to light.

IV. 2. Conserved basic mechanism of COP1 function, but functional divergence of SPA proteins during evolution

SPA genes encode a plant-specific group of proteins, which act as repressors of light signaling together with the COP1 protein in Arabidopsis (Hoecker, 2005). However, COP1 is not plant-specific but is also found in mammals. Mammalian COP1 is not functional in Arabidopsis, in spite of showing nuclear exclusion in response to light as its Arabidopsis homolog suggesting some conserved basic mechanism of action of COP1 in the plant and animal kingdom (Wang et al., 1999). Among plants, COP1 and SPA proteins are well characterized both genetically and biochemically in the dicotyledonous plant Arabidopsis, and it has been shown that these proteins affect almost all stages of plant development in response to light (Laubinger et al., 2004; Laubinger et al; 2006). But the functions of COP1 and SPA proteins, formation of the COP1/SPA complexes as well as their interaction with other light signaling components is completely unknown in other plant species. Here, I identified *COP1* and *SPA* homologs from rice, a monocot, and *Physcomitrella patens*, a moss, and examined their functionality in Arabidopsis.

Database searches identified nine *COP1* homologs in Physcomitrella, whereas only one homolog was present in rice. This is consistent with an earlier phylogeny-based analysis of *COP1* in different plant species (Richardt et al., 2007). It shows that Physcomitrella has acquired and retained several paralogs of *COP1* during evolution. This is in contrast to generally lower amounts of transcription-associated proteins in Physcomitrella as compared to rice and Arabidopsis (Richardt et al., 2007). The expansion of the *COP1* gene family in Physcomitrella is intriguing and may indicate increased importance of COP1 function or its involvement in further specialized functions in this species. While mosses do not etiolate, they certainly possess light responses such as branching of the protonema, phototropism of protonemal filaments, gametophore induction and development, and chloroplast relocation (Wada and Kadota, 1989; Kasahara et al., 2004; Mittmann et al., 2004; Uenaka and Kadota, 2007). The different members of the *COP1* gene family may have specific functions in mediating these diverse light responses in Physcomitrella, thereby representing one possible explanation of the presence of numerous *COP1* genes in moss. Another possibility to explain the multiple *COP1* genes in Physcomitrella is their possible involvement in acquiring UV tolerance, a process that has been shown to be important in mosses (Newsham et al., 2003; Wolf et al., 2010). It has been shown in Arabidopsis that COP1, yet not the SPA proteins, is involved in UV-B tolerance (Oravecz et al., 2006). This UV-B-specific pathway involves the recently described UVR8-COP1-HY5 pathway (Oravecz et al., 2006; Favory et al., 2009). Interestingly, Physcomitrella contains two homologs of

each *UVR8* and *HY5* (Richardt et al., 2007; Rensing et al., 2008). The *CHS* (chalcone synthase) and *PAL* (phenylalanine ammonium lyase) gene families, which mediate molecular responses to UV-B, are also expanded in Physcomitrella in comparison to Arabidopsis. Moreover, Physcomitrella gametophores are more resistant to harmful levels of UV-B radiation when compared to Arabidopsis (Wolf et al., 2010). Taken together, these observations suggest a higher importance of UV-B signaling in Physcomitrella, and the multiple *COP1* genes may contribute to this besides being involved in other light responses.

Rice and Physcomitrella COP1 are highly similar to Arabidopsis COP1, sharing 68% and 62% amino acid identity, respectively. In fact, COP1 from other plant species such as pea and tomato also show more than 60% identity to Arabidopsis COP1, and even the rat homolog shares 44% identity (Richardt et al., 2007). This indicates the importance of COP1 as an E3-ubiquitin ligase in different plant species and even in mammals. Consistent with the observed high sequence similarity, rice and Physcomitrella COP1 were both functional in Arabidopsis (Figure 24, 25). Rice *COP1* complemented all the *cop1-4* mutant phenotypes observed for seedling development, leaf size and flowering time. The examined Physcomitrella *COP1* complemented all the phenotypes of the *cop1-4* mutant except the cotyledon closure phenotype in darkness, which suggests that other Physcomitrella *COP1* genes may be required for this function. Altogether, these results imply that rice and Physcomitrella COP1, in a complex with Arabidopsis SPA proteins, can recognize all substrates of the COP1/SPA complex in Arabidopsis that are involved in de-etiolation, leaf size and flowering time. Further, COP1 may also function as an E3-ubiquitin ligase in rice and Physcomitrella.

Notably, in the present study rice and Physcomitrella *COP1* homologs complemented mutant phenotypes of the Arabidopsis *cop1-4* mutant, a weak *cop1* allele that still retains some residual functions as it expresses a truncated COP1 protein with N-terminal 282 amino acids (McNellis 1994). Therefore, the complementation of *cop1-4* mutant phenotypes may result due to an interaction between the truncated COP1 protein of the *cop1-4* with that of C-terminus of rice and Physcomitrella COP1 homologs. However, in that case also, at least C-terminus of these homologs is functional in Arabidopsis. Hence, in order to test the functionality of rice and Physcomitrella COP1 homologs unequivocally in Arabidopsis, these homologs need to be expressed in the seedling-lethal *cop1-5* mutant.

Contrary to *COP1*, the *SPA* gene-family appears to have expanded during evolution, with only two *SPA* homologs found in Physcomitrella but four in Arabidopsis. This is consistent with a general increase in gene family complexity during evolution. It has already been observed that gene families in Physcomitrella tend to be smaller than

in Arabidopsis (Rensing et al., 2002; 2008). The four members of the *SPA* gene family in Arabidopsis are subdivided into two subclasses based upon sequence similarity: subclass1 having *SPA1* and *SPA2* and subclass2 having *SPA3* and *SPA4*. The four *SPA* genes of Arabidopsis have a certain degree of specificity in their function (Hoecker and Laubinger, 2003; Laubinger et al., 2006). In contrast, the two *SPA* genes of Physcomitrella are highly similar to each other and are more similar to genes belonging to subclass2 of Arabidopsis *SPA*s. Rice, which is intermediate in evolution, also contains two *SPA* genes, of which one is similar to subclass1 and the other one similar to subclass2 of Arabidopsis. This may also point towards the expansion of the *SPA* gene-family during evolution to perform specific functions in higher flowering plants. The importance of gene duplication and neofunctionalization has already been highlighted for evolution of the flower structure (Ferrario et al., 2004; Hernandez-Hernandez et al., 2007). The extant diversity of the orchid perianth has been suggested to be due to gene duplication and functional specification of class B floral homeotic genes (Mondragon-Palomino et al., 2009). In the same pattern, *SPA* genes may also have experienced duplication events during evolution to perform specific functions in higher plants. A possible cause of neofunctionalization after gene duplication might be a change in expression patterns of the respective genes (Mondragon-Palomino et al., 2009). Consistent with this, the four Arabidopsis SPA proteins are expressed at all developmental stages, but with distinct tissue-specific expression patterns (Zhu et al., 2008), suggesting that gene duplication and transcriptional divergence played a role in *SPA* gene family evolution.

Lower sequence homology among the SPA proteins from the three species was observed when compared to that of the COP1 protein, further demonstrating the diversification of the SPA proteins. Relatively less homology among the Arabidopsis and Physcomitrella SPA proteins could be explained by the distant relationship of the two species. However, relatively low homology among the rice and Arabidopsis SPA proteins is intriguing as these two species are more closely related. This further suggests that the *SPA* genes were evolving till very late in evolution. Consistent with the low sequence homology of the rice and Physcomitrella SPA proteins to Arabidopsis SPA proteins, rice and Physcomitrella *SPA*s did not complement any of the Arabidopsis *spa* mutant phenotypes (Figure 27, 28).

Physcomitrella SPAa physically interacted with Arabidopsis COP1 in vitro, suggesting that they may also interact in planta and hence non-functionality of Physcomitrella SPA, and probably rice SPA proteins also, is not due to failure of interaction of these proteins with Arabidopsis COP1 (Figure 30). One reason for the non-functionality of rice and Physcomitrella *SPA* genes in Arabidopsis could be that these

proteins may not be able to fulfill the same function as Arabidopsis SPAs in the COP1/SPA complex. Since Arabidopsis SPA proteins might provide substrate specificity to the COP1/SPA complex, it is possible that rice and Physcomitrella SPA proteins may not recognize the substrates in Arabidopsis, leading to their non-functionality. Interestingly, functional specification of class B floral homeotic gene family in Orchids has also been suggested to involve changes in their target recognition (Mondragon-Palomino et al., 2009). Similar to this, SPA proteins may have different targets in rice and Physcomitrella than in Arabidopsis and, hence, rice and Physcomitrella SPA proteins may not be able to target the substrates of the Arabidopsis SPA proteins. This can be tested by interaction studies of rice and Physcomitrella SPAs with the substrates of the COP1/SPA complex in Arabidopsis.

Non-functionality of rice and Physcomitrella *SPA* genes in Arabidopsis could also be due to a different mechanism of signal transduction through these proteins in rice and Physcomitrella. SPA proteins in those plant species may have altogether different functions with different mechanisms. It is also possible that COP1 does not require SPA proteins for its function early in evolution, as in the case of mammalian COP1, which doesn't require SPA proteins for its function. The in vitro interaction studies showing interaction of Physcomitrella SPA with Physcomitrella COP1, however, suggest the formation of a COP1/SPA complex even in early-evolved plant species (Figure 30). But this complex may still be involved in regulation of different functional processes in these plants than in Arabidopsis. This may be supported by altogether different light responses in Physcomitrella such as branching of the protonema and gametophore induction and development. Thus, the Physcomitrella COP1/SPA complex may regulate these different light responses through entirely different substrates than in Arabidopsis.

Targeted knockdown of genes in Physcomitrella provides a unique facility for reverse genetics and analyzing the functions of individual genes. Therefore, phenotypic characterization of Physcomitrella *spa* knockouts will help to understand the *SPA* gene functions in this species. Further, the molecular characterization of these knockouts will aid to identify SPA targets in Physcomitrella and will, thereby, provide insight into the mechanism of *SPA* gene action early in evolution.

V. Materials and Methods

V. 1. Materials

V. 1. 1. Chemicals and antibiotics

All used chemicals and antibiotics in analytical quality were obtained from Ambion (Austin, USA), Applichem (Dermstadt, Germany), Colgate-Palmolive (Hamburg, Germany), Duchefa (Haarlem, Netherlands), Difco (Detriot, USA), Fluka AG (Buchs, Switezerland), Gibco BRL (Eggenstein, Germany), Invitrogen (Karlsruhe, Germany), MBI Fermentas (St. Leon-Rot, Germany), Merck (Dermstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich (Deisenhofen, Germany).

V. 1. 2. Enzymes, kits, molecular biology materials and radioactivity

Restriction enzymes, dNTPs, PCR enzymes and DNase were obtained from MBI-fermentas (St.Leon-Rot, Germany). Gateway cloning enzymes were acquired from Invitrogen (Karlsruhe, Germany).

^{35}S-methionine was delivered from Amersham Pharmacia Biotech (Uppsala, Sweden).

The following kits were used according to manufacturers' protocols: Plamid Mini and Midi Prep, QIA Gel extraction kit, PCR purification kit and RNeasy Plant Mini Kit (all obtained from Qiagen, GmbH, Hilden, Germany).

V. 1. 3. Oligonucleotides

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

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

Oligonuceotides	Sequences(5'-3')	Application
NcoI F	CATGGGGGCCCGAGCTCC	Expanding the NcoI site in pjTEX-SPA1
NcoI R	CATGGGAGCTCGGGCCCC	Expanding the NcoI site in pjTEX-SPA1
SalI F	TCGACGAGCTCGCGGCCGCG	Expanding the SalI site in pjTEX-SPA1
SalI R	TCGACGCGGCCGCGAGCTCG	Expanding the SalI site in pjTEX-SPA1
pGWB1-F6551	ACACAGCCAGTCTGCAGGTCG	Colony PCR to confirm pGWB1-GUS-SPA1 destination vector
GUS-530R	CATCGCAGCGTAATGCTCTA	Colony PCR to confirm pGWB1-GUS-SPA1 destination vector

CAB3-pro-F	AAAAAGCAGGCTGCAAATCAAGAGAAAATG TGATTCTCGG	*CAB3* promoter entry cloning
CAB3-pro-R	AGAAAGCTGGGTCTGAAACTTTTTGTGTTT TTTTTTTTTTTG	*CAB3* promoter entry cloning
CER6pro F	AAAAAGCAGGCTATCTTCGATATCGGTTGT TG	*CER6* promoter entry cloning
CER6pro R	AGAAAGCTGGGTACGTCGGAGAGTTTTAAT G	CER6 promoter entry cloning
SPA1proF	AAAAAGCAGGCTAAAATAATACAACATGTT GCTGGT	*SPA1* promoter entry cloning
SPA1proR	AGAAAGCTGGGTTTAACAGGCATCAACACT CATT	*SPA1* promoter entry cloning
HinDIII-pSPA1F	AGACTAAGCTTAATAATACAACATGTTGCT GGT	ProSPA1-pGWB1 destination vector generation
HinDIII-pSPA1R	CTAGAAAGCTTTAACAGGCATCAACACTCA TT	ProSPA1-pGWB1 destination vector generation
SdaI-pSPA4F	AGACTCCTGCAGGATGATCTTCTTGGACAT GCATC	ProSPA4-pGWB1 destination vector generation
SdaI-pSPA4R	CTAGACCTGCAGGTGATTACCAAACAAACT CCTCT	ProSPA4-pGWB1 destination vector generation
OsSPA1 GWFP	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCGGGGACGCATGGTTTTCG	*OsSPA1* cDNA entry cloning
OsSPA1 GWRP	GGGGACCACTTTGTACAAGAAAGCTGGGT ATCACACAAGCTCAAGCACTTTAATGC	*OsSPA1* cDNA entry cloning
OsSPA4 GWFP	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGAGGGCTCCCGTGTCGAC	*OsSPA4* cDNA entry cloning
OsSPA4 GWRP	GGGGACCACTTTGTACAAGAAAGCTGGGT ATCAAAAGCTGACATACACCAGGTG	*OsSPA4* cDNA entry cloning
OsCOP1 GWFP	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGGTGACTCGACGGTGGC	*OsCOP1* cDNA entry cloning
OsCOP1 GWRP	GGGGACCACTTTGTACAAGAAAGCTGGGT ATCAAGGAGCAAGTACAAGAACTT	*OsCOP1* cDNA entry cloning
ppSPA17 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTA TATGAAGGAGTTACCAGGCAG	*PpSPAa* cDNA entry cloning
ppSPA17 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT ATCACACCATTTCCAAAATCTTG	*PpSPAa* cDNA entry cloning
ppCOP1 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTA TATGGAGGGAGGAGGTCCTTTC	*PpCOP1* cDNA entry cloning
ppCOP1 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT ATCAGGGAGCAAGGACCAAGAC	*PpCOP1* cDNA entry cloning
AtSPA1-RT-F	GTGTTTTTCGAGGGGTTGTG	*AtSPA1* transgene expression
AtSPA1-RT-R	GACCAGGCTCTCTTCTGGTG	*AtSPA1* transgene expression
AtSPA4-RT-F	GGTCGGTCTTTAGCATTTGG	*AtSPA4* transgene expression

AtSPA4-RT-R	TGGAAATGCCTTGTGGTACA	*AtSPA4* transgene expression
OsSPA1-RT-F	CTTTTGGCTCGGCTGATTAT	*OsSPA1* transgene expression
OsSPA1-RT-R	GGCATGGGAAAGGTTTTGTA	*OsSPA1* transgene expression
OsSPA4-RT-F	TTCTTTGCAACTGCTGGTGT	*OsSPA4* transgene expression
OsSPA4-RT-R	CATCACTCCCGCTAACCAAT	*OsSPA4* transgene expression
PpSPA-RT-F	CAAGACCAAGGCCAACGTAT	*PpSPAa* transgene expression
PpSPA-RT-R	ATTCGTTTCCGATCCACAAG	*PpSPAa* transgene expression
Act2-F	ACTTTCATCCAGCCGTTTTGA	Control for RT-PCR
Act2-R	ACGATTGGTTGAATATCATCAG	Control for RT-PCR

V. 1. 4. Bacterial strains

For standard cloning, *Escherichia coli* strain *DH5*α was used. For Gateway cloning of destination vectors, the *ccdB* gene resistant *Escherichia coli* strain *DB3.1* (Invitrogen) was used. *Agrobacterium tumefaciens* strain *GV3101 (pMP90RK)* was used for all plant transformations.

V. 1. 5. Cloning vectors

Entry vector pDONR221 was used for BP reactions to clone promoters and cDNAs.

Binary destination vector pGWB1 (Kanamycin and Hygromycin resistance genes, Nakagawe et al., 2007) with *GUS-SPA1* gene fusion cloned after the gateway recombination site was used for expressing *GUS-SPA1* under the control of different tissue-specific promoters.

Destination vectors pGJ2169 GW containing *35S* promoter before the gateway cassette (Spectinomycin and Basta resistance, kindly provided by Prof. George Coupland) was used for expressing Arabidopsis, rice and Physcomitrella *COP1* genes.

Binary destination vector pGWB1 (Kanamycin and Hygromycin resistance genes) with *SPA1* or *SPA4* native promoter cloned before the gateway recombination site were used for expressing Arabidopsis, rice and Physcomitrella *SPA* genes.

pDEST14 (ampicilin resistance, Invitrogen) and pJIC39 (ampicilin resistance, kindly provided by Prof. George Coupland) were used to generate bait and prey constructs, respectively, for in vitro coimmunoprecipitation assay.

V. 1. 6. Plant lines

The *spa1 spa3 spa4* triple mutant was used as a background to study the complementation of adult plant phenotypes and the *spa1 spa2 spa3* mutant was used to study the complementation of seedling and stomata differentiation phenotype for tissue-specific expression of GUS-SPA1. These mutants were derived from a cross of *spa1-3 spa2-1* with *spa3-1 spa4-1* as described previously (Laubinger et al., 2004).

The *spa1 spa3 spa4* triple mutant was used as a background to study the functionality of rice and Physcomitrella *SPA* genes.

The *cop1-4* mutant (McNellis et al., 1994) was used a background to study the functionality of rice and Physcomitrella COP1 genes.

The *phyB*, *cry2*, *spa1 phyB* and *spa1 cry2* mutants were used for epistatic analysis. All these mutants are described previously (Reed et al., 1993; Parks et al., 2001; Guo et al., 1998)

V. 2. Methods

V. 2. 1. Cloning

Conventional DNA cloning was performed by using standard protocols as described in Sambrook and Russell, 2001. Conventional cloning was employed to clone a *GUS-SPA1* in the binary destination vector pGWB1 to express *GUS-SPA1* under different tissue-specific promoters. *SPA1* and *SPA4* native promoters were also cloned in the pGWB1 through conventional cloning to express rice and Physcomitrella *SPA* genes.

All other cloning were performed employing Gateway cloning. BP and LR reaction were performed according to manufacture's protocol (Invitrogen).

Details of cloning strategies are explained in V. 3.

V. 2. 2. Bacterial transformation and plasmid isolation

Escherichia coli competent cells were transformed by heat shock method and then cells were plated on selective media and kept at 37^0C overnight. Agrobacteria cells were transformed using electroporation and then cells were plated on LB media with appropriate antibiotics and incubated at 28^0C for two days.

Plasmid DNA from *E. coli* in miniprep or midiprep scale was isolated using Plasmid minikit or midikit (Qiagen) following manufacturer's instructions.

V. 2. 3. Plasmid DNA manipulations

Correctness of PCR generated cloned DNA fragments was determined by appropriate

restriction enzyme digestion followed by sequencing (AGOWA, Berlin and GATC, Konstanz) and University of Cologne (Department of Genetics). The sequence alignment analysis was performed using Vector NTIsuite software (Invitrogen). Constructs were also designed by using Vector NTIsuite software (Invitrogen).

V. 2. 4. Plant transformation

Agrobacterium-mediated transformation of flowering Arabidopsis plants was performed using floral-dip method as described previously (Clough and Bent, 1998)

V. 2. 5. Seed sterilization

For sterile growth of Arabidopsis on MS-plates, seeds were surface sterilized. For liquid sterilization 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.

For dry seed sterilization, aliquots of seeds were incubated with chlorine gas. To produce chlorine gas, 80 ml of sodium hypochlorite was mixed with 2.5 ml of concentrated hydrochloric acid in an exsiccator. Aliquots of seeds were incubated for approximately 4 hours.

V. 2. 6. 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. After that plates were transferred either to darkness or kept for 21 h in the dark at 21°C and were then exposed to Rc for 3 days. Light conditions were generated using LED light sources (Quantum Devices, Barneveld, WI, USA).

To determine the flowering time or leaf size in controlled conditions, seeds were sown directly onto soil and plants were grown in a randomized fashion in SD (8 hours light/16 hours darkness) or in LD (16 hours light/8 hours darkness) at 21°C. The light sources were fluorescent tubes (110 µmol m^{-2} s^{-1}).

V. 2. 7. Handling transgenic plants

T1 seeds were screened with the appropriate selective antibiotics.

For *tissue specific promoter:GUS-SPA1* transgenic plants, GUS-staining of the transgenic leaves as well as preliminary phenotypic analysis of leaf size and flowering time was performed in T1. Transgenic lines were characterized in details for correct tissue-specific expression of GUS-SPA1 in T2. Three independent transgenic lines showing correct tissue-specific expression of GUS-SPA1 were propagated to obtain homozygous lines. These were subsequently used for detailed phenotypic analysis.

For rice and Physcomitrella *COP1* and *SPA* transgenic plants, complementation analysis of leaf size and flowering time was performed in T1 with several independent transgenic plants. Analysis of complementation of seedling phenotype was performed in T2.

V. 2. 8. GUS histochemistry and microscopy

GUS activity was determined as described previously with some minor modifications (Jefferson et al., 1987).

Plant tissues to be analyzed for GUS activity were vacuum infiltrated in GUS-staining buffer for complete infiltration of the substrate (X-Gluc). Two times infiltration was performed for approximately 15 minutes. Tissues were incubated for 4 hours or overnight at 37°C. Reaction was stopped by adding 70% ethanol. Chlorophyll was removed by several washing steps with 70% ethanol at 37^0C.

GUS-staining solution:	0.5 mM	NaPO4 (pH 7.0)
	10 mM	EDTA (pH 7.0)
	1.0 mM	potassium-ferrocyanide ($K4Fe(CN)_6$+ H2O)
	1.0 mM	potassium ferricyanide ($K3Fe(CN)_6$))
	1 mM	5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-Gluc)
	0.1%	TritonX-100

GUS-stained samples were then documented using a digital camera (Nikon D5000) for whole plants or binocular (Nikon SMZ-U) for seedlings or individual leaves.

V. 2. 9. Sectioning and microscopy

Tissue-specificity of GUS-SPA1 in leaves and stems of transgenic plants was determined by free-hand sections. Hand sections of the leaf or stem tissues were prepared by slicing with a razor blade before staining and destaining as described above. Later the GUS-stained sections were observed and photographed under compound light microscope (Nikon E800).

Tissue-specificity at the seedling stage was determined by GUS-staining of the seedlings followed by Technovit embedding and cross-sectioning by microtome. GUS-stained seedlings were fixed in a buffer containing 50% Ethanol, 5% Acetic acid and 3% Formaldehyde. Fixed seedlings were dehydrated in graded ethanol series (70%, 96% and 100%; 2 hour each), embedded in Technovit 7100 (Heraeus Kulzer) according to the manufacturer's protocol and sectioned using microtome. Tissue sections were mounted on slides and examined with a compound light microscope (Nikon E800).

V. 2. 10. 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 Microsoft excel software program.

V. 2. 11. Quantification of leaf size

Leaf size was determined by measuring the lengths of the longest leaf (petiole and leaf blade) of 3-week-old plants under long day and 4-week-old plants under short day. 15 plants were analyzed for each homozygous line under both long and short day in case of *tissue-specific pro:GUS-SPA1* transgenic plants. The leaf size of *SUC2:GUS-SPA1+/- CAB3:GUS-SPA1+/-* double transgenic plants was determined in F1. Leaf size of rice and Physcomitrella *COP1* and *SPA* transgenic plants were measured in T1. Statistical analyses were made using Microsoft excel software program.

V. 2. 12. Quantification of flowering time

Time of flowering under short days or long days was determined by counting the numbers of true leaves at that day when first inflorescence was visible by eye. Additionally number of days to flower from the day of sowing was also determined. For *tissue-specific pro:GUS-SPA1* transgenic plants, 15 plants were analyzed for each genotype. For rice and Physcomitrella *COP1* and *SPA* transgenic plants, flowering time of several independent T1 plants was determined. Statistical analyses were made using Microsoft excel software program.

V. 2. 13. Preparation of cotyledons for stomata and pavement cell analysis

Cotyledons of ten-day-old dark-grown seedlings were used to analyze the stomata and pavement cell differentiation. Cotyledon samples were preserved in 95% ethanol, rehydrated in a graded ethanol series, and placed in the clear solution (glycerol: chloral

hydrate: water, 1:8:1) overnight. Samples mounted in the clear solution were visualized using a compound light microscope (Nikon E800).

V. 2. 14. RNA isolation and RT-PCR

For amplification of Physcomitrella *COP1* and *SPA* cDNAs, total RNA was isolated form 100mg of Physcomitrella gametophores 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. Subsequently, cDNAs were synthesized using Transcriptor high fidelity cDNA synthesis kit (Roche, Mannheim, germany) according to manufacturer's instruction. Later, desired cDNAs were amplified using specific primers and high fidelity *pfu* DNA polymerase.

For RT-PCR to examine the transgene expression in rice and Physcomitrella *SPA* transgenic lines, total RNA was from 10-day-old light-grown seedlings 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, 2µl cDNA was used as template. Arabidopsis, rice and Physcomitrella *SPA* gene fragments were amplified using gene-specific primers. The *ACT2* fragment was used as a control. RT-PCR products were analyzed on agarose gels after 30 PCR cycles. Amplicon sizes for different *SPA* genes from Arabidopsis, rice and Physcomitrella were: *AtSPA1* (380bp), *AtSPA4* (333bp), *OsSPA1* (331bp), *OsSPA4* (301bp) and *PpSPAa* (355bp).

V. 2. 15. In vitro interaction assay

Bait and prey proteins were synthesized in the presence of ^{35}S-methionine using the reticulocyte in vitro transcription/translation system, TnT (Promega, Madison, WI, USA), according to manufacturer's instructions. For the in vitro interaction assay, 10 µl from each TnT-produced bait and prey proteins were incubated in 200 µl binding buffer (20mM Tris pH7.5, 150mM NaCl, 1mM DTT and 0.1% Tween-20) with protease-inhibitors (Complete, EDTA-free, Roche, Mannheim, Germany) at 4^{0}C for 2-3 h while rotating. Subsequently, 1 µg of monoclonal antibody against GAD (Santa Cruz Biotechnology, CA, USA) was added and the reaction was incubated for one additional hour. After adding 10 µl protein A-coated magnetic beads (Dynal, Oslo, Norway) and incubation for another hour, the magnetic beads were washed four times with 800 µl

binding buffer. Supernatant and pellet fractions were analyzed by SDS-PAGE with 10% acrylamide and visualized using a phosphorimager (Fuji).

V. 2. 16. Bioinformatics and sequence alignment

The CLUSTAL W program was used for alignment and comparison of Arabidopsis, rice and Physcomitrella COP1 and SPA homologs. All the sequence alignments were carried out as well as phylogenetic tree was generated using online resources at http://www.ebi.ac.uk/Tools/clustalw2/index.html. Physcomitrella COP1 and SPA homologs sequences were retrieved using http://www.cosmoss.org/ database and rice COP1 and SPA homologs sequences were retrieved from http://cdna01.dna.affrc.go.jp/.

V.3. Cloning strategies

V. 3. 1. *Tissue-specific promoter:GUS-SPA1* constructs

i) Generation of pGWB1-GUS-SPA1 destination vector:

pjTEX-SPA1 (Kindly provided by Prof. Ute Hoecker) plasmid was used to excise the GUS-SPA1 for later cloning into pGWB1 (Figure 34A). *GUS-SPA1* was flanked with NcoI and SalI enzymes in the pjTEX-SPA1. The NcoI restriction site was expanded by a polylinker (NcoI – ApaI – SacI – NcoI) and the SalI site was expanded by polylikner (SalI – SacI – NotI – BamHI). Later, *GUS-SPA1* fusion was released from the modified pjTEX-SPA1 through partial Sac I digestion (as *SPA1* has one internal Sac I site). Afterwards, Sac I digested *GUS-SPA1* fragment was cloned into the unique Sac I site of pGWB1, which is after the Gateway cassette, to generate the pGWB1-GUS-SPA1 destination vector (Figure 34B). Later, the tissue-specific promoters were cloned into the Gateway cassette to express the *GUS-SPA1* fusion.

ii) Tissue-specific promoters entry clones:

ProSUC2; *ProRolC*; *ProKNAT1*; *ProML1* and *ProTobRB7* entry clones were kindly provided by Prof. George Coupland (Max Planck Institute, Cologne) (An et al., 2004). All these promoter entry clones were in pDONR207 entry vector.

I cloned *ProSPA1*; *ProCAB3* and *ProCER6* in pDONR221 entry vector. The promoter fragments were amplified from Arabidopsis wild type genomic DNA using sequence-specific primers with attached attB1 site to forward primer and attB2 site to reverse primer. The purified PCR product was cloned into pDONR221 entry vector through BP reaction. Figure 34C shows a representative promoter entry clone harboring *ProSPA1*.

iii) Generation of *Pro:GUS-SPA1* constructs:

Pro:GUS-SPA1 constructs were generated through a LR reaction between the created pGWB1-GUS-SPA1 destination vector and different promoter entry clones. The promoters got cloned into the gateway cassette of the destination vector, and thus different constructs expressing *GUS-SPA1* under the control of the different tissue-specific promoters were developed. A representative expression clone expressing *GUS-SPA1* under the control of native *SPA1* promoter is shown in Figure 34D.

Later, these constructs were introduced in Arabidopsis *spa* mutant backgrounds through Agrobacterium-mediated floral dip transformation (Clough and Bent, 1998).

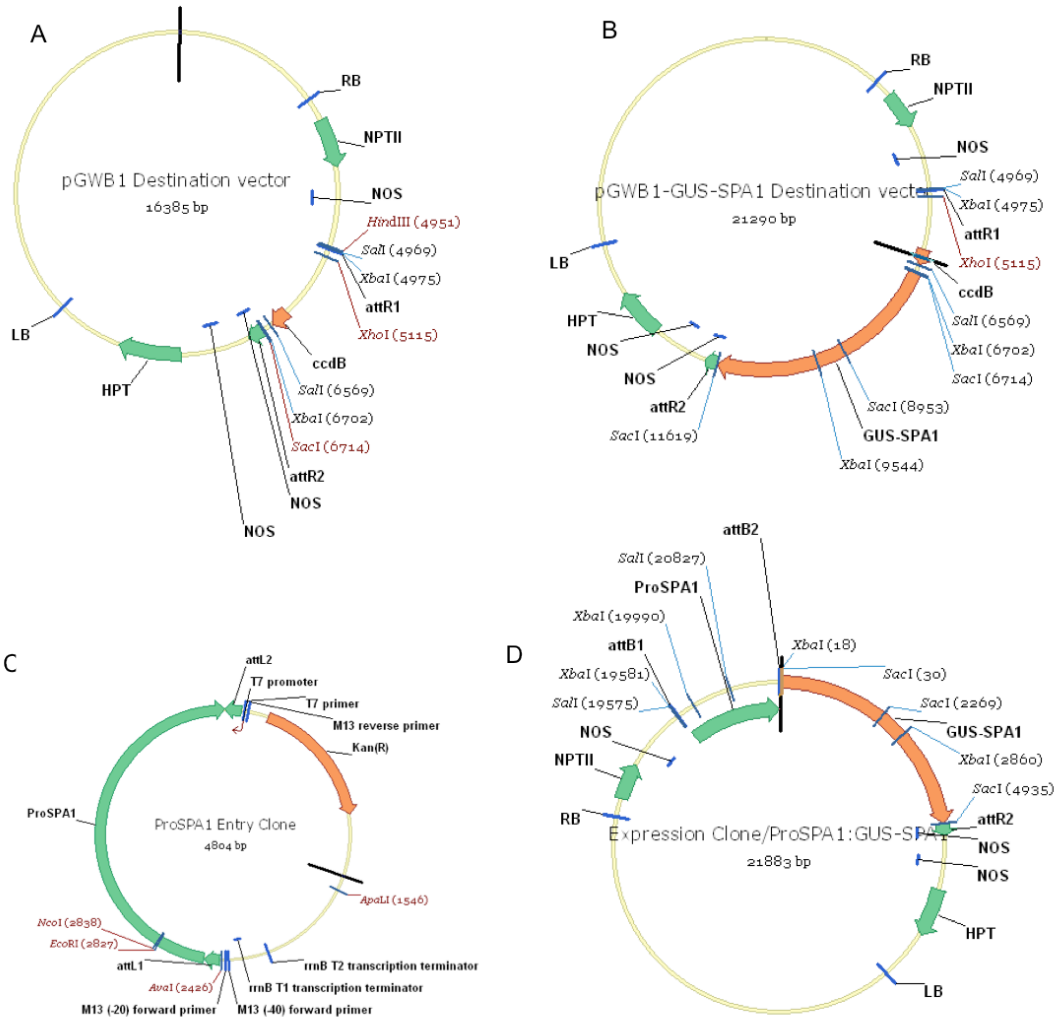

Figure 34: Generation of *tissue-specific promoter:GUS-SPA1* constructs.

(A) pGWB1 destination vector.
(B) pGWB1-GUS-SPA1 destination vector created by cloning *GUS-SPA1* fusion in unique Sac I site of pGWB1 vector.
(C) Representative promoter entry clone containing *ProSPA1*.
(D) Representative expression clone expressing *GUS-SPA1* under the native *SPA1* promoter. Similarly, other expression clones also expressed *GUS-SPA1* under different tissue-specific promoters.

V. 3. 2. *35S:AtCOP1/OsCOP1/PpCOP1* constructs

OsCOP1 and *PpCOp1* cDNA were amplified using gene specific primers with attached attB sites. *OsCOP1* was amplified from a full-length cDNA clone obtained from NIAS, Japan. *PpCOP1* was amplified from cDNA synthesized from Physcomitrella gametophores. The amplified cDNA sequences were cloned into pDONR221 entry vector. For *AtCOP1*, an existing entry clone was used. Subsequently, the entry clones were recombined with pGJ2169 GW destination vector (Kindly provided by Prof. George Coupland) containing *35S* promoter before the Gateway cassette. Thus, the destination clones expressing *AtCOP1*, *OsCOP1* or *PpCOp1* under the *35S* promoter were generated.

V. 3. 3. *pSPA1:AtSPA1/OsSPA1/PpSPAa* and *pSPA4:AtSPA4/OsSPA4/ PpSPAa* constructs

First *ProSPA1* was cloned into pGWB1 destination vector using unique Hind III site and *ProSPA4* was cloned using unique SdaI site (Figure 35A and B). These modified pGWB1 destination vectors have Gateway cassette after the *ProSPA1* or *ProSPA4* promoter. *OsSPA1* and *OsSPA4* cDNAs were amplified from full-length cDNA clones obtained from NIAS, Japan. Physcomitrella *SPA* was amplified from cDNA synthesized from the Physcomitrella gametophores. Existing Arabidopsis *AtSPA1* and *AtSPA4* entry clones were used. Modified ProSPA1-pGWB1 destination vector was recombined with *AtAPA1*, *OsSPA1* or *PpSPAa* entry clones to generate *pSPA1:AtSPA1/OsSPA1/PpSPAa* clones. Similarly, modified ProSPA4-pGWB1 destination vector was recombined with *AtAPA4*, *OsSPA4* or *PpSPAa* entry clones to generate *pSPA4:AtSPA4/OsSPA4/PpSPAa* clones.

V. 3. 4. Constructs for in vitro interaction studies

Destination clones carrying *T7:AtCOP1* and *T7:GAD-AtSPA1* was kindly provided by Alexender Maier. Destination clone carrying *T7:PpCOP1* was generated by recombining the *PpCOP1* entry clone with pDEST14 destination vector (Invitrogen) via LR reaction. Similarly, *T7:GAD-PpSPAa* was generated by recombining the *PpSPAa* entry clone with pJIC39 destination vector (Kindly provided by Prof. George Coupland). Subsequently, these constructs were used for coimmunoprecipitation studies.

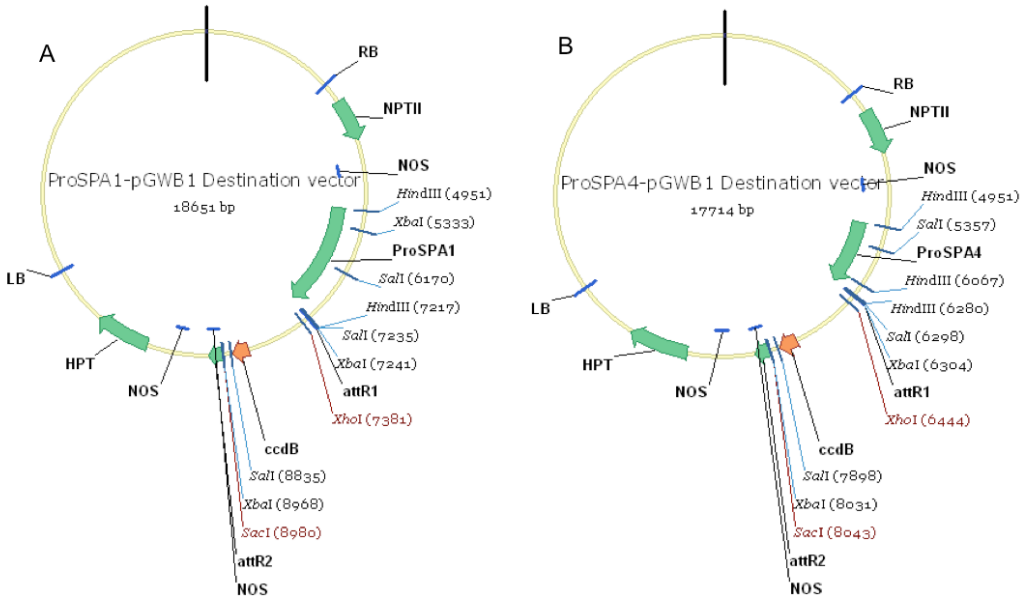

Figure 35: Maps of destination vectors expressing Arabidopsis, rice and Physcomitrella *SPA* genes.

(A) ProSPA1-pGWB1 destination vector.
(B) ProSPA4-pGWB1 destination vector.

VI. Supplement

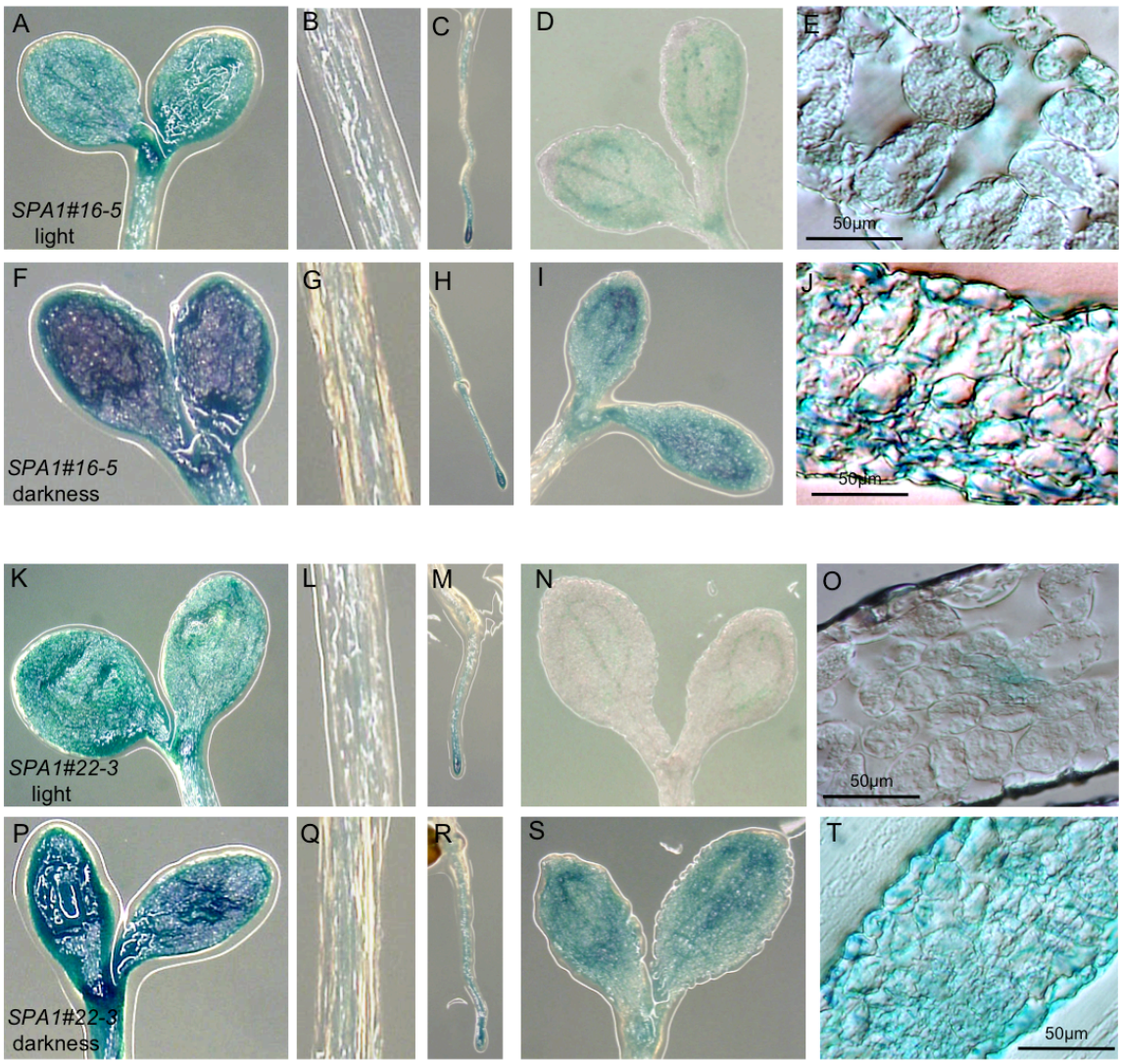

Figure S1: GUS-SPA1 accumulation in seedlings of two additional homozygous transgenic *spa1 spa2 spa3* mutant expressing *GUS-SPA1* under the control of the native *SPA1* promoter.

(A) – **(E)** Light-grown *SPA1:GUS-SPA1#16-5* transgenic seedlings.
(F) – **(J)** Dark-grown *SPA1:GUS-SPA1#16-5* transgenic seedlings.
(K) – **(O)** Light-grown *SPA1:GUS-SPA1#22-3* transgenic seedlings.
(P) – **(T)** Dark-grown *SPA1:GUS-SPA1#22-3* transgenic seedlings.
Cotyledons (A, F, K, P), hypocotyls (B, G, L, Q) and roots (C, H, M, R) of transgenic seedlings stained overnight for GUS activity; Cotyledons after 4 hours of GUS-staining (D, I, N, S); Cross-section of overnight GUS-stained cotyledons (E, J, O, T).

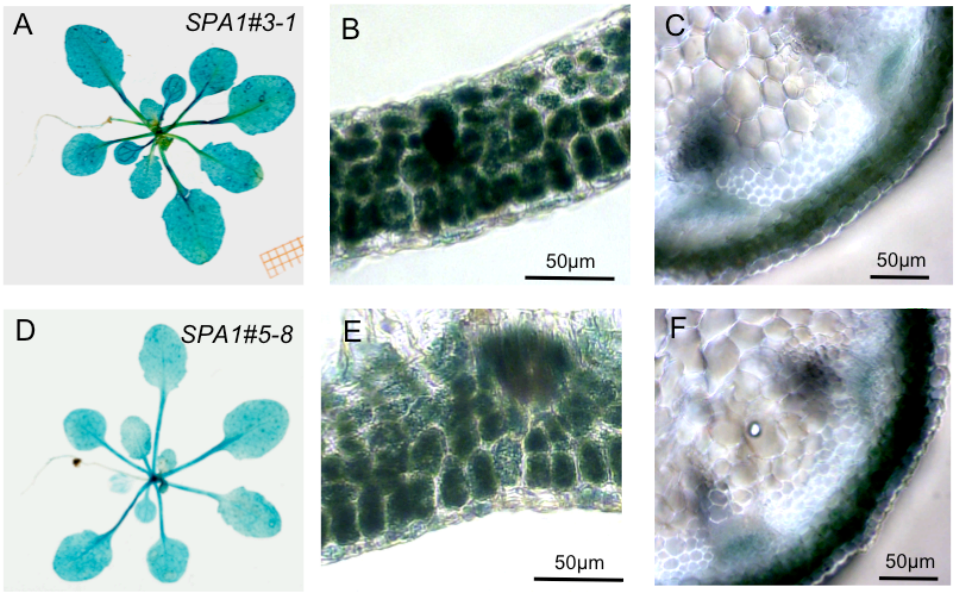

Figure S2: GUS-staining pattern in of two additional homozygous transgenic *spa1 spa3 spa4* plant expressing *GUS-SPA1* under the control of the native *SPA1* promoter.
(A) – **(C)** *SPA1:GUS-SPA1#3-1*
(D) – **(F)** *SPA1:GUS-SPA1#5-8*
3-week-old long-day-grown transgenic plants (A, D), Free-hand cross-sections through leaves of transgenic plants (B, E), Free-hand cross-section of the stem of a transgenic plant (C, F).

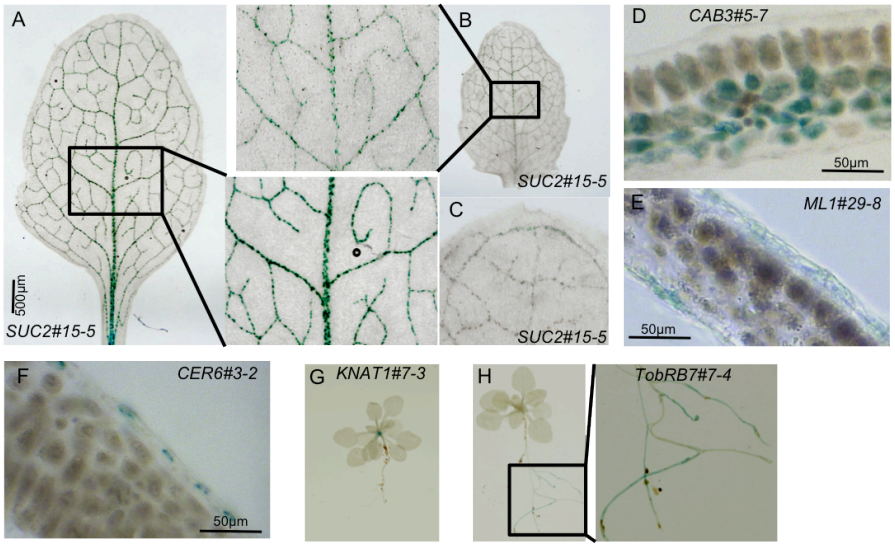

Figure S3: Tissue-specific expression of GUS-SPA1 in homozygous transgenic *spa1 spa3 spa4* plants expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters.
(A) – **(C)** Matured (A), developing (B) and young (C) leaves of a transgenic *SUC2:GUS-SPA1#15-5* plant.
(D) – **(F)** cross section through a leaf of *CAB3:GUS-SPA1#5-7*(D), *ML1:GUS-SPA1#29-8*(E) and *CER6:GUS-SPA1#3-2*(F) plants.
(G) – **(H)** A 3-week-old transgenic *KNAT1:GUS-SPA1#7-3*(G) and *TobRB7:GUS-SPA1# 7-4*(H)plants.

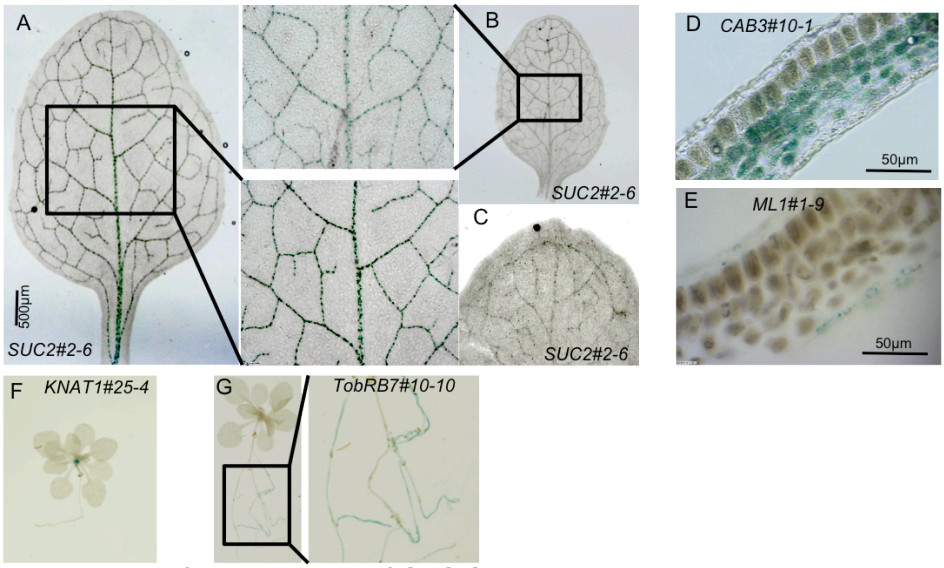

Figure S4: Tissue-specific expression of GUS-SPA1 in homozygous transgenic *spa1 spa3 spa4* plants expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters.
(A) – **(C)** Matured (A), developing (B) and young (C) leaves of a transgenic *SUC2:GUS-SPA1#2-6* plant.
(D) – **(E)** cross section through a leaf of *CAB3:GUS-SPA1#10-1*(D) and *ML1:GUS-SPA1#1-9*(E) plants.
(F) – **(G)** A 3-week-old transgenic *KNAT1:GUS-SPA1#25-4*(F) and *TobRB7:GUS-SPA1# 10-10*(G)plant.

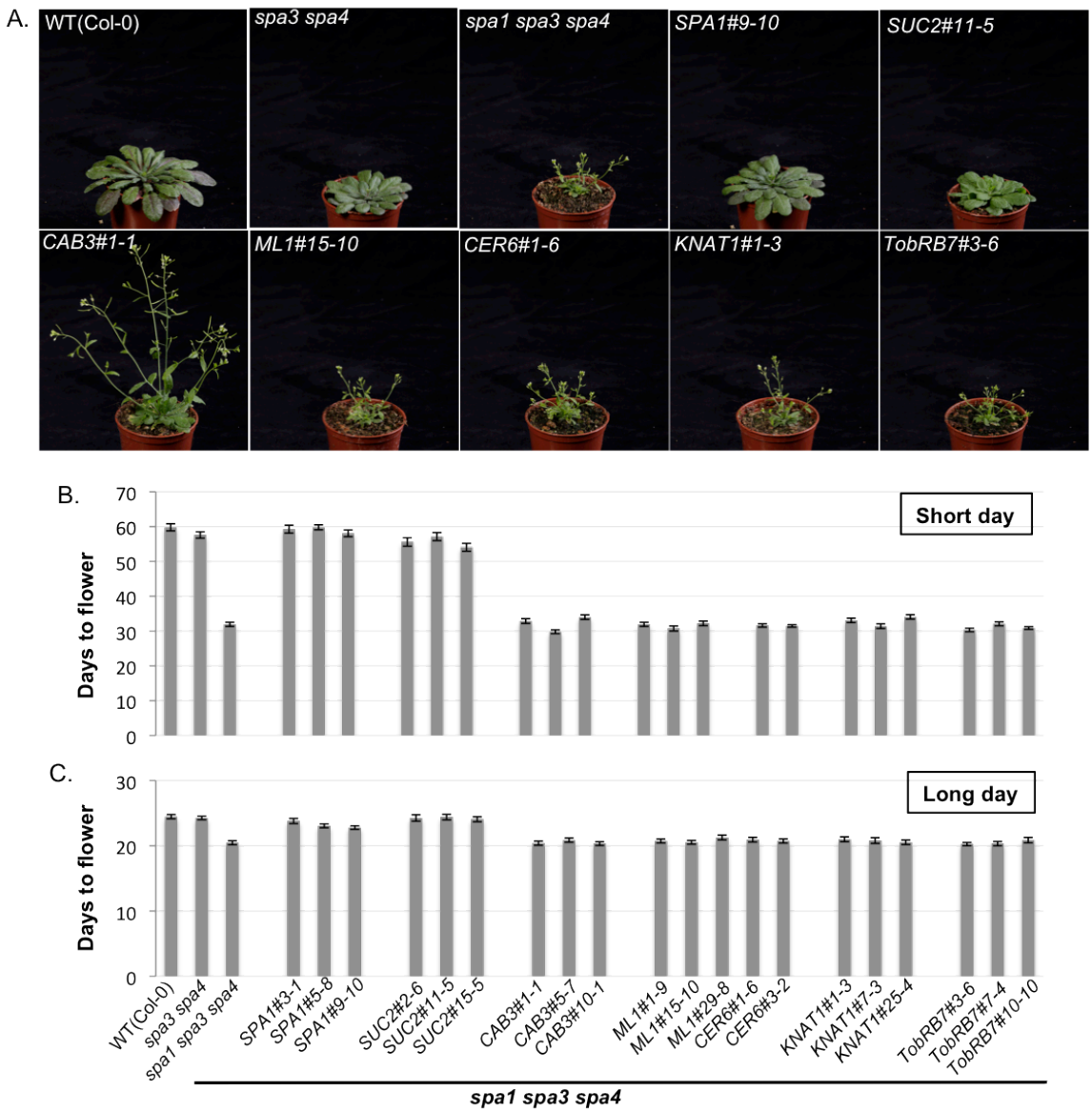

Figure S5: SPA1 acts in the phloem to regulate flowering time.

(A) Visual phenotype of the one representative transgenic plant for each transgene, grown in short days for 50 days. As controls, wild-type (WT), *spa3 spa4* and *spa1 spa3 spa4* mutant plants are shown. Transgenic plants are denoted as the promoters used to drive the *GUS-SPA1* expression.
(B) and **(C)** Quantification of flowering time as number of days to flower of homozygous transgenic plants expressing GUS-SPA1 in specific tissues in the *spa1 spa3 spa4* mutant in short days (B) and long days (C). Transgenic lines are denoted as the promoters used to drive the *GUS-SPA1* expression. At least 15 plants were analyzed per genotype. Error bars denote the standard error of the mean.

Figure S6: Visual phenotype of 4-week-old short-day-grown homozygous transgenic *spa1 spa3 spa4* mutant plants expressing GUS-SPA1 in specific tissues. Transgenic plants are denoted as the promoters used to drive the *GUS-SPA1* expression. As controls, wild-type (WT), *spa3 spa4* and *spa1 spa3 spa4* mutant plants are shown.

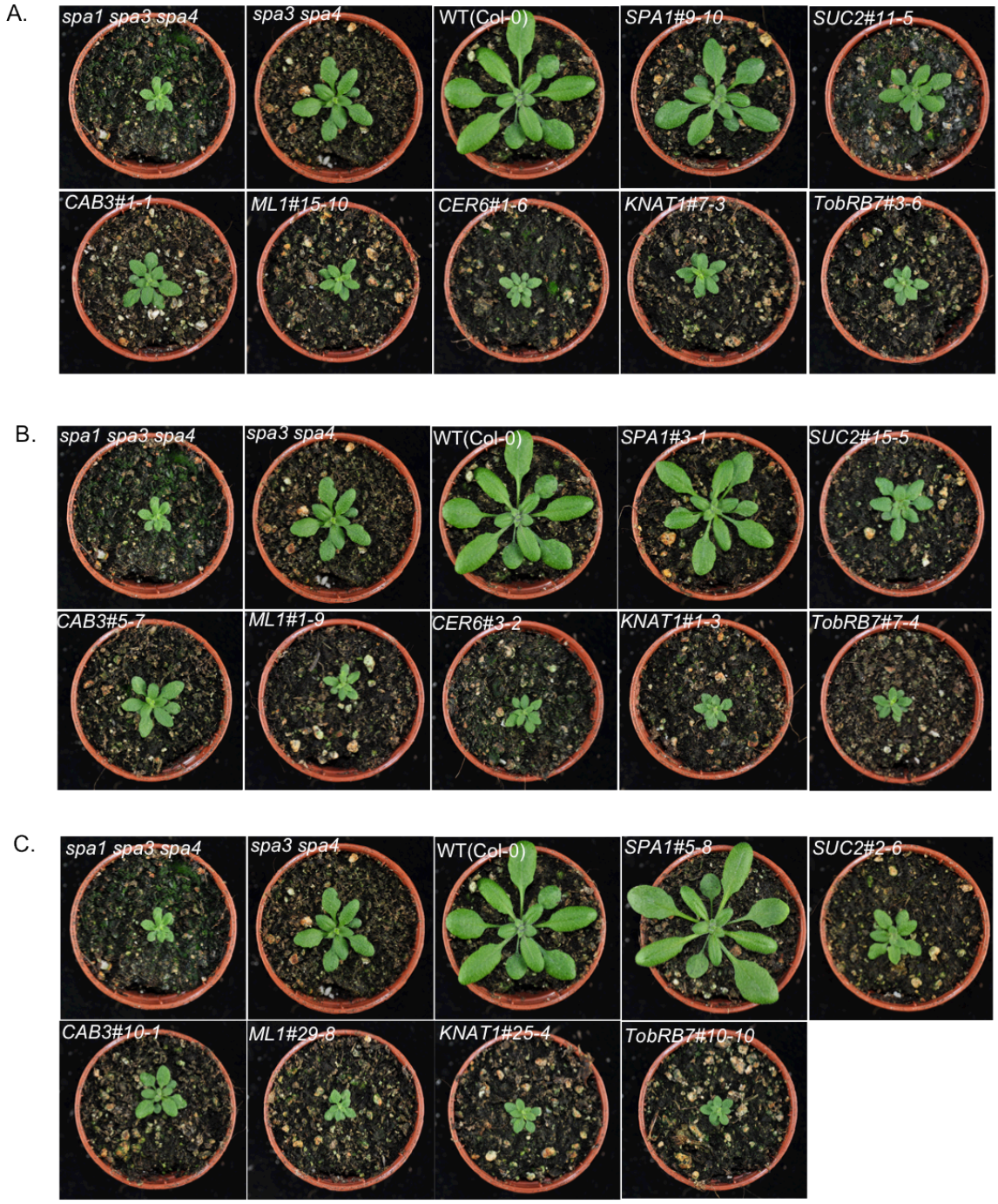

Figure S7: Visual phenotype of 3-week-old long-day-grown homozygous transgenic *spa1 spa3 spa4* mutant plants expressing GUS-SPA1 in specific tissues. Transgenic plants are denoted as the promoters used to drive the *GUS-SPA1* expression. As controls, wild-type (WT), *spa3 spa4* and *spa1 spa3 spa4* mutant plants are shown.

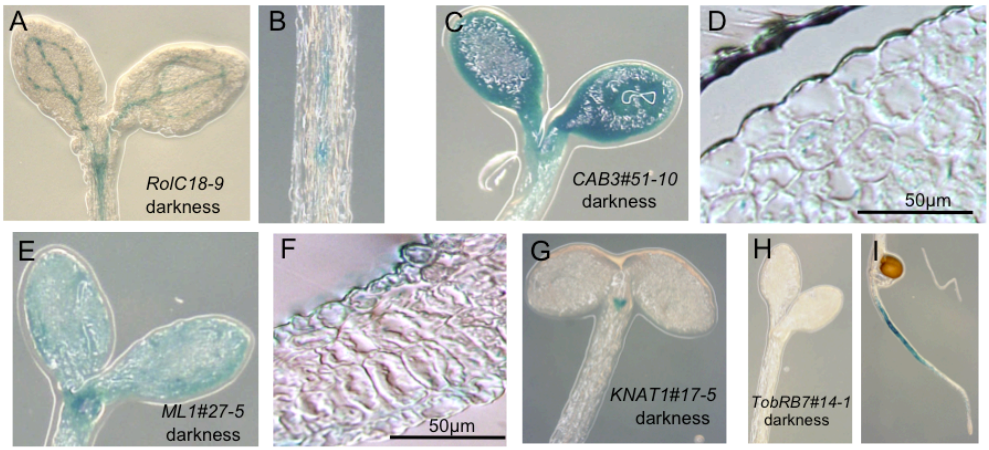

Figure S8: Tissue-specific expression of GUS-SPA1 in dark-grown transgenic *spa1 spa2 spa3* seedlings expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters. Seedlings were grown in darkness for four days and stained overnight for GUS activity.
(A) – **(B)** Cotyledons (A) and the hypocotyl (B) of a transgenic *RolC:GUS-SPA1#18-9* seedling.
(C) – **(D)** Cotyledons and the hypocotyl (C) and cross-section of a cotyledon (D) of a transgenic *CAB3:GUS-SPA1#51-10* seedling .
(E) – **(F)** Cotyledons and the hypocotyl (E) and cross-section of a cotyledon (F) of a transgenic *ML1:GUS-SPA1#27-5* seedling.
(G) Cotyledons and the hypocotyl of a transgenic *KNAT1:GUS-SPA1#17-5* seedling.
(H) – **(I)** Cotyledons, the hypocotyl (H) and the root (I) of a transgenic *TobRB7:GUS-SPA1#14-1* seedling.

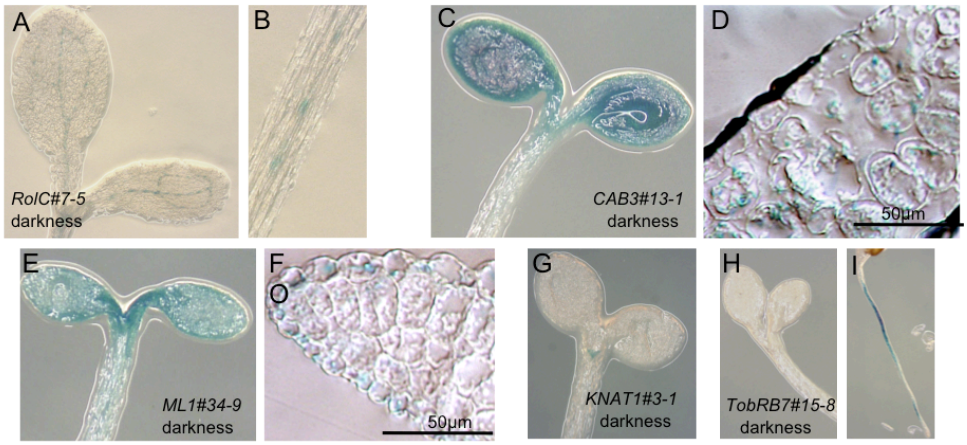

Figure S9: Tissue-specific expression of GUS-SPA1 in dark-grown transgenic *spa1 spa2 spa3* seedlings expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters. Seedlings were grown in darkness for four days and stained overnight for GUS activity.
(A) – **(B)** Cotyledons (A) and the hypocotyl (B) of a transgenic *RolC:GUS-SPA1#7-5* seedling.
(C) – **(D)** Cotyledons and the hypocotyl (C) and cross-section of a cotyledon (D) of a transgenic *CAB3:GUS-SPA1#13-1* seedling .
(E) – **(F)** Cotyledons and the hypocotyl (E) and cross-section of a cotyledon (F) of a transgenic *ML1:GUS-SPA1#34-9* seedling.
(G) Cotyledons and the hypocotyl of a transgenic *KNAT1:GUS-SPA1#3-1* seedling.
(H) – **(I)** Cotyledons, the hypocotyl (H) and the root (I) of a transgenic *TobRB7:GUS-SPA1#15-8* seedling.

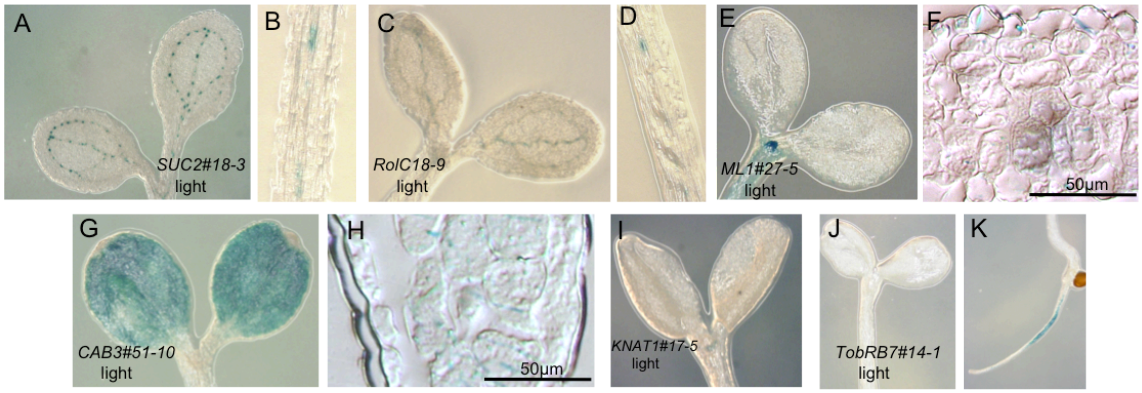

Figure S10: Tissue-specific expression of GUS-SPA1 in light-grown transgenic *spa1 spa2 spa3* seedlings expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters. Seedlings were grown in red-light (0.1 µmol m^{-2} s^{-1}) for four days and stained overnight for GUS activity.
(A) – **(B)** Cotyledons (A) and the hypocotyl (B) of a transgenic *SUC2:GUS-SPA1#18-3* seedling.
(C) – **(D)** Cotyledons (A) and the hypocotyl (B) of a transgenic *RolC:GUS-SPA1#18-9* seedling.
(E) – **(F)** Cotyledons and the hypocotyl (E) and cross-section of a cotyledon (F) of a transgenic *ML1:GUS-SPA1#*27-5 seedling.
(G) – **(H)** Cotyledons and the hypocotyl (C) and cross-section of a cotyledon (D) of a transgenic *CAB3:GUS-SPA1#51-10* seedling.
(I) Cotyledons and the hypocotyl of a transgenic *KNAT1:GUS-SPA1#17-5* seedling.
(J) – **(K)** Cotyledons, the hypocotyl (H) and the root (I) of a transgenic *TobRB7:GUS-SPA1# 14-1* seedling.

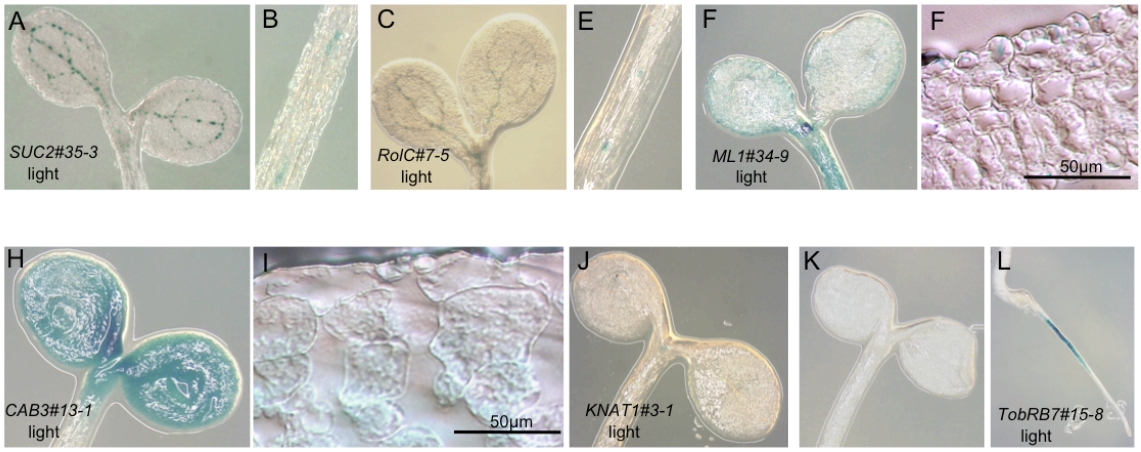

Figure S11: Tissue-specific expression of GUS-SPA1 in light-grown transgenic *spa1 spa2 spa3* seedlings expressing *GUS-SPA1* under the control of different tissue-specifically expressed promoters. Seedlings were grown in red-light (0.1 µmol m^{-2} s^{-1}) for four days and stained overnight for GUS activity.
(A) – **(B)** Cotyledons (A) and the hypocotyl (B) of a transgenic *SUC2:GUS-SPA1#35-3* seedling.
(C) – **(D)** Cotyledons (A) and the hypocotyl (B) of a transgenic *RolC:GUS-SPA1#7-5* seedling.
(E) – **(F)** Cotyledons and the hypocotyl (E) and cross-section of a cotyledon (F) of a transgenic *ML1:GUS-SPA1#34-9* seedling.
(G) – **(H)** Cotyledons and the hypocotyl (C) and cross-section of a cotyledon (D) of a transgenic *CAB3:GUS-SPA1#13-1* seedling.
(I) Cotyledons and the hypocotyl of a transgenic *KNAT1:GUS-SPA1#3-1* seedling.
(J) – **(K)** Cotyledons, the hypocotyl (H) and the root (I) of a transgenic *TobRB7:GUS-SPA1#15-8* seedling.

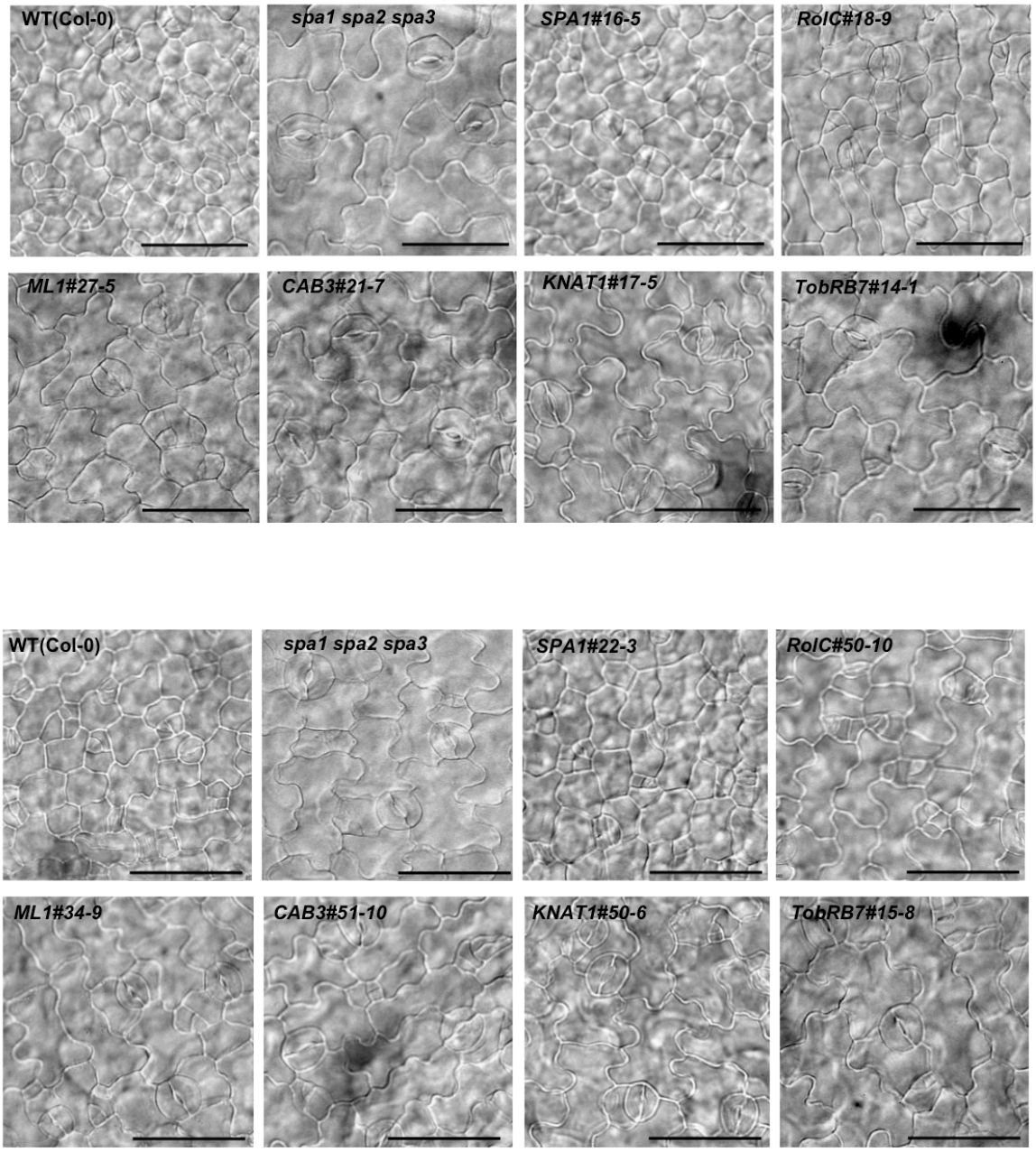

Figure S12: Abaxial cotyledon epidermis of 10-day-old dark-grown seedlings of wild-type, *spa1 spa2 spa3* and two additional homozygous transgenic lines expressing *GUS-SPA1* under each tissue-specific promoter in the *spa1 spa2 spa3* mutant. Transgenic lines are denoted as the promoters used to drive the *GUS-SPA1* expression in each picture.

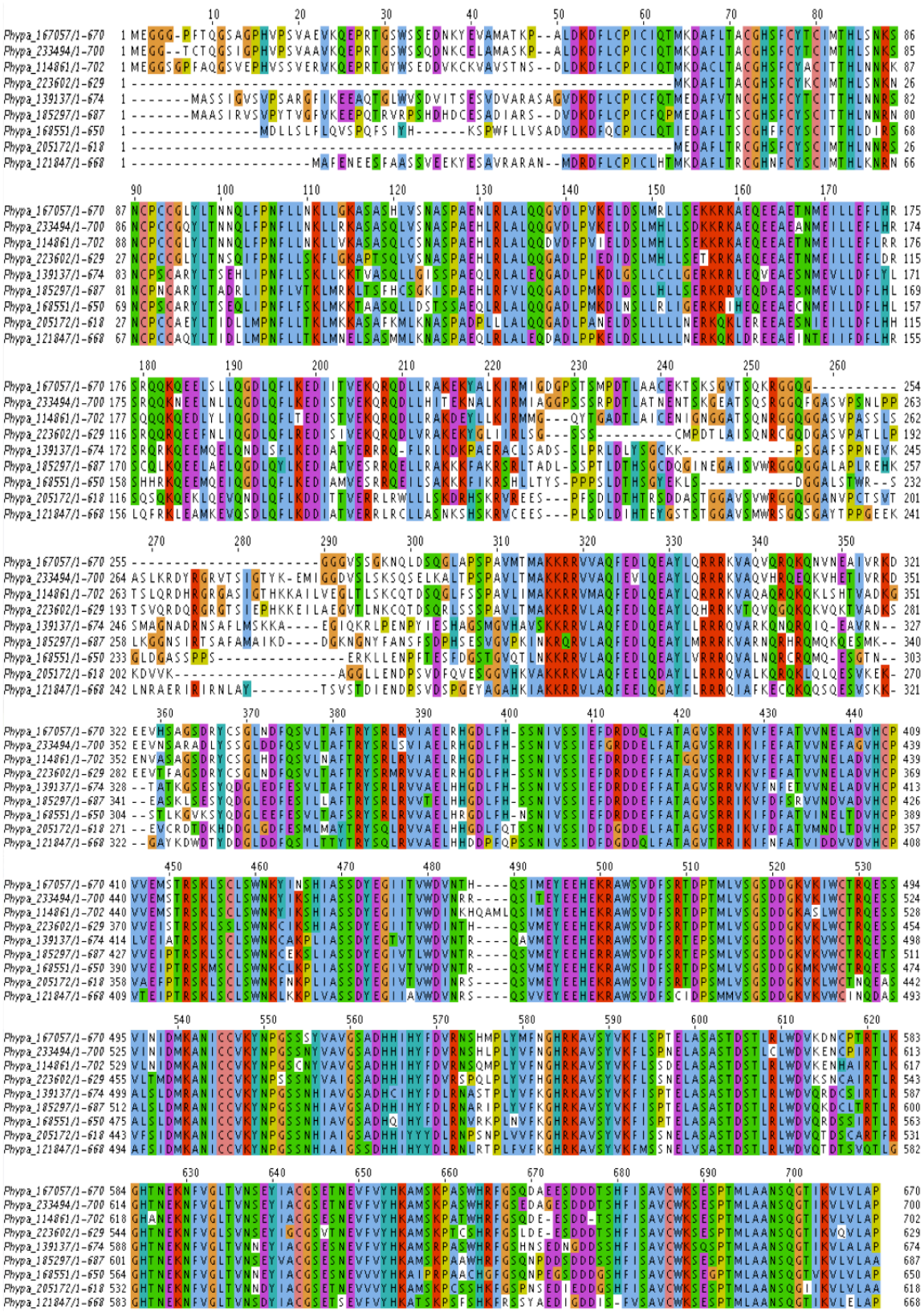

Figure S13: Sequence alignment of nine Physcomitrella COP1 protein sequences. Phypa_167057 was used in present study for the complementation of the Arabidopsis *cop1* mutant phenotype.

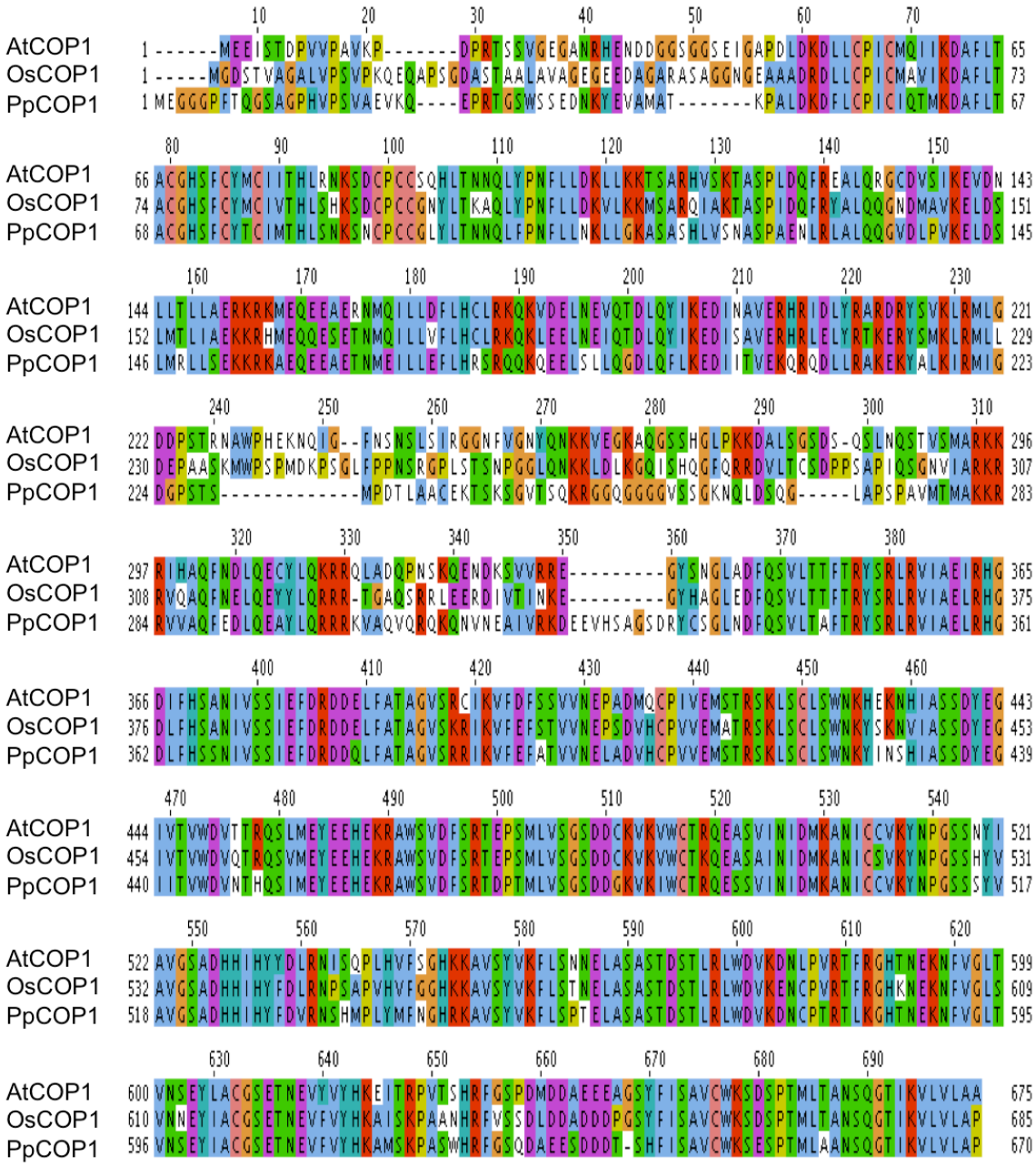

Figure S14: Alignment of AtCOP1, OsCOP1 and PpCOP1 protein sequences.

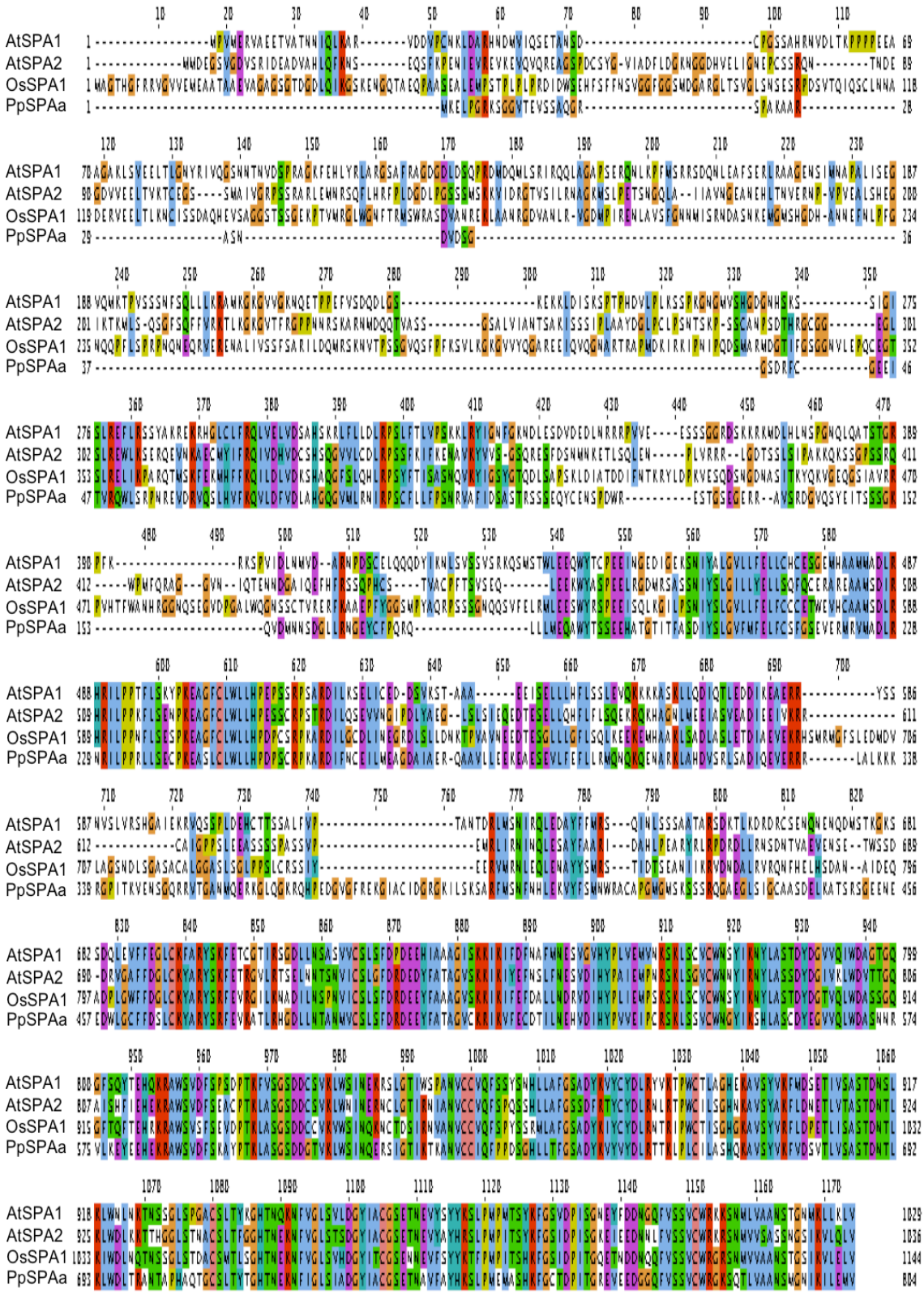

Figure S15: Alignment of AtSPA1, AtSPA2, OsSPA1 and PpSPAa protein sequences.

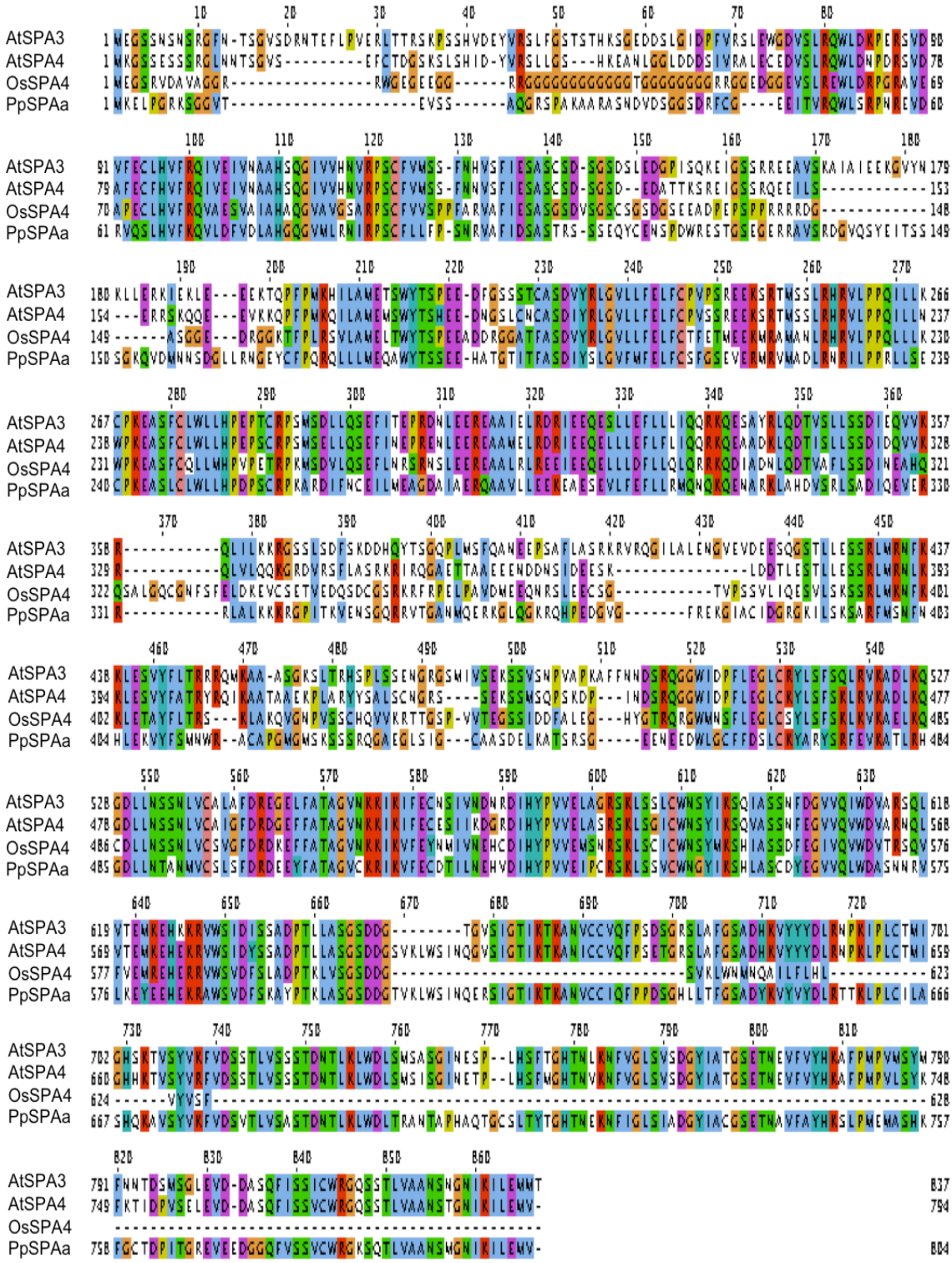

Figure S16: Alignment of AtSPA3, AtSPA4, OsSPA4 and PpSPAa protein sequences.

VII. References

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., and Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science **309,** 1052-1056.

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

Ahmad, M., and Cashmore, A.R. (1997). The blue-light receptor cryptochrome 1 shows functional dependence on phytochrome A or phytochrome B in *Arabidopsis thaliana*. Plant J **11,** 421-427.

Ahmad, M., Jarillo, J.A., Smirnova, O., and Cashmore, A.R. (1998). The CRY1 blue light photoreceptor of Arabidopsis interacts with phytochrome A in vitro. Mol Cell **1,** 939-948.

Ahmad, M., Lin, C., and Cashmore, A.R. (1995). Mutations throughout an Arabidopsis blue-light photoreceptor impair blue-light-responsive anthocyanin accumulation and inhibition of hypocotyl elongation. Plant J **8,** 653-658.

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

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

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

Ang, L.H., Chattopadhyay, S., Wei, N., Oyama, T., Okada, K., Batschauer, A., and Deng, X.W. (1998). Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of Arabidopsis development. Mol Cell **1,** 213-222.

Bae, G., and Choi, G. (2008). Decoding of light signals by plant phytochromes and their interacting proteins. Annu Rev Plant Biol **59,** 281-311.

Bai, Y., Falk, S., Schnittger, A., Jakoby, M.J., and Huelskamp, M. (2010). Tissue layer specific regulation of leaf length and width in Arabidopsis as revealed by the cell autonomous action of ANGUSTIFOLIA. Plant J **61,** 191-199.

Bauer, D., Viczian, A., Kircher, S., Nobis, T., Nitschke, R., Kunkel, T., Panigrahi, K.C., Adam, E., Fejes, E., Schafer, E., and Nagy, F. (2004). Constitutive photomorphogenesis 1 and multiple photoreceptors control degradation of phytochrome interacting factor 3, a transcription factor required for light signaling in Arabidopsis. Plant Cell **16,** 1433-1445.

Bischoff, F., Millar, A.J., Kay, S.A. and Furuya, M. (1997). Phytochrome-induced intercellular signalling activates *cab::luciferase* gene expression. Plant J **12,** 839-849.

Biswas, K.K., Neumann, R., Haga, K., Yatoh, O., and Iino, M. (2003). Photomorphogenesis of rice seedlings: a mutant impaired in phytochrome-mediated inhibition of coleoptile growth. Plant Cell Physiol **44,** 242-254.

Black, M. and Shuttleworth, J.E. (1974). The role of the cotyledons in the photocontrol of hypocotyl extension in *Cucumis sativus* L. Planta **117,** 57-66.

Bohlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A.M., Jansson, S., Strauss, S.H., and Nilsson, O. (2006). CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. Science **312,** 1040-1043.

Booker, J., Chatfield, S., and Leyser, O. (2003). Auxin acts in xylem-associated or medullary cells to mediate apical dominance. Plant Cell **15,** 495-507.

Bou-Torrent, J., Roig-Villanova, I., and Martinez-Garcia, J.F. (2008). Light signaling: back to space. Trends Plant Sci **13,** 108-114.

Briggs, W.R., Beck, C.F., Cashmore, A.R., Christie, J.M., Hughes, J., Jarillo, J.A., Kagawa, T., Kanegae, H., Liscum, E., Nagatani, A., Okada, K., Salomon, M., Rudiger, W., Sakai, T., Takano, M., Wada, M., and Watson, J.C. (2001). The phototropin family of photoreceptors. Plant Cell **13,** 993-997.

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

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

Brown, B.A., and Jenkins, G.I. (2008). UV-B signaling pathways with different fluence-rate response profiles are distinguished in mature Arabidopsis leaf tissue by requirement for UVR8, HY5, and HYH. Plant Physiol **146,** 576-588.

Carabelli, M., Possenti, M., Sessa, G., Ciolfi, A., Sassi, M., Morelli, G., and Ruberti, I. (2007). Canopy shade causes a rapid and transient arrest in leaf development through auxin-induced cytokinin oxidase activity. Genes Dev **21,** 1863-1868.

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

Casal, J.J. (2002). Environmental cues affecting development. Curr Opin Plant Biol **5,** 37-42.

Casal, J.J., and Sanchez, R.A. (1998). Phytochromes and seed germination. Seed Sci. Res. **8,** 317-329.

Casal, J.J. and Smith, H. (1988). The loci of perception for phytochrome control of internode growth in light-grown mustard: promotion by low phytochrome photoequilibria in the internode is enhanced by blue light perceived by the leaves. Planta **176,** 277-282.

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

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

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

Chaudhury, A.M., Letham, S., Craig, S., and Dennis, E.S. (1993). *amp1* - a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J **4,** 907-916.

Chen, M., Chory, J., and Fankhauser, C. (2004). Light signal transduction in higher plants. Annu Rev Genet **38,** 87-117.

Chory, J., Chatterjee, M., Cook, R.K., Elich, T., Fankhauser, C., Li, J., Nagpal, P., Neff, M., Pepper, A., Poole, D., Reed, J., and Vitart, V. (1996). From seed germination to flowering, light controls plant development via the pigment phytochrome. Proc Natl Acad Sci U S A **93,** 12066-12071.

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

Chory, J., Reinecke, D., Sim, S., Washburn, T., and Brenner, M. (1994). A Role for Cytokinins in De-Etiolation in Arabidopsis (det Mutants Have an Altered Response to Cytokinins). Plant Physiol **104,** 339-347.

Christie, J.M. (2007). Phototropin blue-light receptors. Annu Rev Plant Biol **58,** 21-45.

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

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

Cluis, C.P., Mouchel, C.F., and Hardtke, C.S. (2004). The Arabidopsis transcription factor HY5 integrates light and hormone signaling pathways. Plant J **38,** 332-347.

Colon-Carmona, A., Chen, D.L., Yeh, K.C., and Abel, S. (2000). Aux/IAA proteins are phosphorylated by phytochrome in vitro. Plant Physiol **124,** 1728-1738.

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

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

Cove, D., Bezanilla, M., Harries, P., and Quatrano, R. (2006). Mosses as model systems for the study of metabolism and development. Annu Rev Plant Biol **57,** 497-520.

De Greef, J.A. and Caubergs, R. (1972). Interorgan correlations and phytochrome: hypocotyl hook opening. Arch Int Physiol Biochim **80,** 959-960.

de Ruijter, N.C.A., Verhees, J., van Leeuwen, W., and van der Krol, A.R. (2003). Evaluation and Comparison of the GUS, LUC and GFP reporter system for gene expression studies in plants. Plant Biol. **5,** 103-115.

Deng, X.W., Caspar, T., and Quail, P.H. (1991). *cop1*: a regulatory locus involved in light-controlled development and gene expression in Arabidopsis. Genes Dev **5,** 1172-1182.

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

Deng, X.W., and Quail, P. (1992). Genetic and phenotypic characterization of *cop1* mutants of Arabidopsis thaliana. The Plant Journal **2,** 83-95.

Devlin, P.F., Halliday, K.J., Harberd, N.P., and Whitelam, G.C. (1996). The rosette habit of *Arabidopsis thaliana* is dependent upon phytochrome action: novel phytochromes control internode elongation and flowering time. Plant J **10,** 1127-1134.

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

Devlin, P.F., Patel, S.R., and Whitelam, G.C. (1998). Phytochrome E influences internode elongation and flowering time in Arabidopsis. Plant Cell **10,** 1479-1487.

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

Duek, P.D., and Fankhauser, C. (2005). bHLH class transcription factors take centre stage in phytochrome signalling. Trends Plant Sci **10,** 51-54.

Endo, M., Mochizuki, N., Suzuki, T., and Nagatani, A. (2007). CRYPTOCHROME2 in vascular bundles regulates flowering in Arabidopsis. Plant Cell **19,** 84-93.

Endo, M., Nakamura, S., Araki, T., Mochizuki, N., and Nagatani, A. (2005). Phytochrome B in the mesophyll delays flowering by suppressing *FLOWERING LOCUS T* expression in Arabidopsis vascular bundles. Plant Cell **17,** 1941-1952.

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

Favory, J.J., Stec, A., Gruber, H., Rizzini, L., Oravecz, A., Funk, M., Albert, A., Cloix, C., Jenkins, G.I., Oakeley, E.J., Seidlitz, H.K., Nagy, F., and Ulm, R. (2009). Interaction of COP1 and UVR8 regulates UV-B-induced photomorphogenesis and stress acclimation in Arabidopsis. EMBO J **28,** 591-601.

Ferrario, S., Immink, R.G., and Angenent, G.C. (2004). Conservation and diversity in flower land. Curr Opin Plant Biol **7,** 84-91.

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

Fleming, A.J., McQueen-Mason, S., Mandel, T., and Kuhlemeier, C. (1997). Induction of leaf primordia by the cell wall protein expansin. Science **276,** 1415-1418.

Franklin, K.A. (2008). Shade avoidance. New Phytol **179,** 930-944.

Franklin, K.A., Larner, V.S., and Whitelam, G.C. (2005). The signal transducing photoreceptors of plants. Int J Dev Biol **49,** 653-664.

Franklin, K.A., Praekelt, U., Stoddart, W.M., Billingham, O.E., Halliday, K.J., and Whitelam, G.C. (2003). Phytochromes B, D, and E act redundantly to control multiple physiological responses in Arabidopsis. Plant Physiol **131,** 1340-1346.

Franklin, K.A., and Quail, P.H. (2010). Phytochrome functions in Arabidopsis development. J Exp Bot **61,** 11-24.

Franklin, K.A., and Whitelam, G.C. (2004). Light signals, phytochromes and cross-talk with other environmental cues. J Exp Bot **55,** 271-276.

Fukaki, H., Nakao, Y., Okushima, Y., Theologis, A. and Tasaka, M. (2005). Tissue-specific expression of stabilized SOLITARY-ROOT/IAA14 alters lateral root development in Arabidopsis. Plant J **44,** 382-395.

Gendreau, E., Traas, J., Desnos, T., Grandjean, O., Caboche, M., and Hofte, H. (1997). Cellular basis of hypocotyl growth in Arabidopsis thaliana. Plant Physiol **114,** 295-305.

Gonzalez, N., De Bodt, S., Sulpice, R., Jikumaru, Y., Chae, E., Dhondt, S., Van Daele, T., De Milde, L., Weigel, D., Kamiya, Y., Stitt, M., Beemster, G.T., and Inze, D. (2010). Increased leaf size: different means to an end. Plant Physiol **153,** 1261-1279.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. (2001). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. Nature **414,** 271-276.

Griffiths, S., Dunford, R.P., Coupland, G., and Laurie, D.A. (2003). The evolution of *CONSTANS-like* gene families in barley, rice, and Arabidopsis. Plant Physiol **131,** 1855-1867.

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

Halliday, K.J., Martinez-Garcia, J.F., and Josse, E.M. (2009). Integration of light and auxin signaling. Cold Spring Harb Perspect Biol **1,** a001586.

Hayama, R., Agashe, B., Luley, E., King, R., and Coupland, G. (2007). A circadian rhythm set by dusk determines the expression of *FT* homologs and the short-day photoperiodic flowering response in Pharbitis. Plant Cell **19,** 2988-3000.

Hayama, R., and Coupland, G. (2004). The molecular basis of diversity in the photoperiodic flowering responses of Arabidopsis and rice. Plant Physiol **135,** 677-684.

Hennig, L., Stoddart, W.M., Dieterle, M., Whitelam, G.C., and Schafer, E. (2002). Phytochrome E controls light-induced germination of Arabidopsis. Plant Physiol **128,** 194-200.

Henriques, R., Jang, I.C., and Chua, N.H. (2009). Regulated proteolysis in light-related signaling pathways. Curr Opin Plant Biol **12,** 49-56.

Hernandez-Hernandez, T., Martinez-Castilla, L.P., and Alvarez-Buylla, E.R. (2007). Functional diversification of B MADS-box homeotic regulators of flower development: Adaptive evolution in protein-protein interaction domains after major gene duplication events. Mol Biol Evol **24,** 465-481.

Hirose, F., Shinomura, T., Tanabata, T., Shimada, H., and Takano, M. (2006). Involvement of rice cryptochromes in de-etiolation responses and flowering. Plant Cell Physiol **47,** 915-925.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., and Sakakibara, H. (2008). Regulation of cytokinin biosynthesis, compartmentalization and translocation. J Exp Bot **59,** 75-83.

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

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

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

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

Hooker, T.S., Millar, A.A., and Kunst, L. (2002). Significance of the expression of the CER6 condensing enzyme for cuticular wax production in Arabidopsis. Plant Physiol **129,** 1568-1580.

Hsu, C.Y., Liu, Y., Luthe, D.S., and Yuceer, C. (2006). Poplar FT2 shortens the juvenile phase and promotes seasonal flowering. Plant Cell **18,** 1846-1861.

Imaizumi, T., Kadota, A., Hasebe, M., and Wada, M. (2002). Cryptochrome light signals control development to suppress auxin sensitivity in the moss *Physcomitrella patens*. Plant Cell **14,** 373-386.

Imaizumi, T., and Kay, S.A. (2006). Photoperiodic control of flowering: not only by coincidence. Trends Plant Sci **11,** 550-558.

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

Imlau, A., Truernit, E., and Sauer, N. (1999). Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell **11,** 309-322.

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

Izawa, T., Oikawa, T., Sugiyama, N., Tanisaka, T., Yano, M., and Shimamoto, K. (2002). Phytochrome mediates the external light signal to repress FT orthologs in photoperiodic flowering of rice. Genes Dev **16,** 2006-2020.

Izawa, T., Takahashi, Y., and Yano, M. (2003). Comparative biology comes into bloom: genomic and genetic comparison of flowering pathways in rice and Arabidopsis. Curr Opin Plant Biol **6,** 113-120.

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

Jang, I.C., Henriques, R., Seo, H.S., Nagatani, A., and Chua, N.H. (2010). Arabidopsis PHYTOCHROME INTERACTING FACTOR Proteins Promote Phytochrome B Polyubiquitination by COP1 E3 Ligase in the Nucleus. Plant Cell.

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

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

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

Jenkins, G.I. (2009). Signal transduction in responses to UV-B radiation. Annu Rev Plant Biol **60,** 407-431.

Jensen, P.J., Hangarter, R.P., and Estelle, M. (1998). Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown Arabidopsis. Plant Physiol **116,** 455-462.

Jiao, Y., Ma, L., Strickland, E., and Deng, X.W. (2005). Conservation and divergence of light-regulated genome expression patterns during seedling development in rice and Arabidopsis. Plant Cell **17,** 3239-3256.

Johnson, E., Bradley, M., Harberd, N.P., and Whitelam, G.C. (1994). Photoresponses of Light-Grown *phyA* Mutants of Arabidopsis (Phytochrome A Is Required for the Perception of Daylength Extensions). Plant Physiol **105,** 141-149.

Jung, J.H., Seo, Y.H., Seo, P.J., Reyes, J.L., Yun, J., Chua, N.H., and Park, C.M. (2007). The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in Arabidopsis. Plant Cell **19,** 2736-2748.

Kang, C.Y., Lian, H.L., Wang, F.F., Huang, J.R., and Yang, H.Q. (2009). Cryptochromes, phytochromes, and COP1 regulate light-controlled stomatal development in Arabidopsis. Plant Cell **21,** 2624-2641.

Kang, X., and Ni, M. (2006). Arabidopsis SHORT HYPOCOTYL UNDER BLUE1 contains SPX and EXS domains and acts in cryptochrome signaling. Plant Cell **18,** 921-934.

Kasahara, M., Kagawa, T., Sato, Y., Kiyosue, T., and Wada, M. (2004). Phototropins mediate blue and red light-induced chloroplast movements in *Physcomitrella patens*. Plant Physiol **135,** 1388-1397.

Kasahara, M., Swartz, T.E., Olney, M.A., Onodera, A., Mochizuki, N., Fukuzawa, H., Asamizu, E., Tabata, S., Kanegae, H., Takano, M., Christie, J.M., Nagatani, A., and Briggs, W.R. (2002). Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and *Chlamydomonas reinhardtii*. Plant Physiol **129,** 762-773.

Kehr, J., and Buhtz, A. (2008). Long distance transport and movement of RNA through the phloem. J Exp Bot **59,** 85-92.

Kendrick, R.E., and Kronenberg, G.H.M. (1994). Photomorphogenesis in land plants (2nd edn) Kluwer Academic Rordrecht, The Netherlands.

Kim, J.Y., Yuan, Z., Cilia, M., Khalfan-Jagani, Z., and Jackson, D. (2002). Intercellular trafficking of a KNOTTED1 green fluorescent protein fusion in the leaf and shoot meristem of Arabidopsis. Proc Natl Acad Sci U S A **99,** 4103-4108.

Kim, T.H., Kim, B.H., and von Arnim, A.G. (2002). Repressors of photomorphogenesis. Int Rev Cytol **220,** 185-223.

Kim, W.Y., Fujiwara, S., Suh, S.S., Kim, J., Kim, Y., Han, L., David, K., Putterill, J., Nam, H.G., and Somers, D.E. (2007). ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. Nature **449,** 356-360.

Kinoshita, T., Doi, M., Suetsugu, N., Kagawa, T., Wada, M., and Shimazaki, K. (2001). Phot1 and phot2 mediate blue light regulation of stomatal opening. Nature **414,** 656-660.

Kircher, S., Gil, P., Kozma-Bognar, L., Fejes, E., Speth, V., Husselstein-Muller, T., Bauer, D., Adam, E., Schafer, E., and Nagy, F. (2002). Nucleocytoplasmic partitioning of the plant photoreceptors phytochrome A, B, C, D, and E is regulated differentially by light and exhibits a diurnal rhythm. Plant Cell **14,** 1541-1555.

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

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., and Yano, M. (2002). *Hd3a*, a rice ortholog of the Arabidopsis *FT* gene, promotes transition to flowering downstream of Hd1 under short-day conditions. Plant Cell Physiol **43,** 1096-1105.

Kozuka, T., Kobayashi, J., Horiguchi, G., Demura, T., Sakakibara, H., Tsukaya, H., and Nagatani, A. (2010). Involvement of auxin and brassinosteroid in the regulation of petiole elongation under the shade. Plant Physiol **153,** 1608-1618.

Lake, J.A., Quick, W.P., Beerling, D.J., and Woodward, F.I. (2001). Plant development. Signals from mature to new leaves. Nature **411,** 154.

Lariguet, P., and Dunand, C. (2005). Plant photoreceptors: phylogenetic overview. J Mol Evol **61,** 559-569.

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

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

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

Ledger, S., Strayer, C., Ashton, F., Kay, S.A., and Putterill, J. (2001). Analysis of the function of two circadian-regulated *CONSTANS-LIKE* genes. Plant J **26,** 15-22.

Leu, W.M., Cao, X.L., Wilson, T.J., Snustad, D.P., and Chua, N.H. (1995). Phytochrome A and phytochrome B mediate the hypocotyl-specific downregulation of TUB1 by light in arabidopsis. Plant Cell **7,** 2187-2196.

Leyser, H.M., Pickett, F.B., Dharmasiri, S., and Estelle, M. (1996). Mutations in the *AXR3* gene of Arabidopsis result in altered auxin response including ectopic expression from the *SAUR-AC1* promoter. Plant J **10,** 403-413.

Li, Q.H., and Yang, H.Q. (2007). Cryptochrome signaling in plants. Photochem Photobiol **83,** 94-101.

Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., Alvarez, J.P., and Eshed, Y. (2006). The tomato *FT* ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. Proc Natl Acad Sci U S A **103,** 6398-6403.

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

Lin, C., and Shalitin, D. (2003). Cryptochrome structure and signal transduction. Annu Rev Plant Biol **54,** 469-496.

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

Lin, R. and Wang, H. (2007). Targeting proteins for degradation by Arabidopsis COP1: Teamwork is what matters. J Integr Plant Biol **49,** 35-42.

Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K., and Hake, S. (1994). A *knotted1-like* homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell **6,** 1859-1876.

Liscum, E., and Briggs, W.R. (1995). Mutations in the NPH1 locus of Arabidopsis disrupt the perception of phototropic stimuli. Plant Cell **7,** 473-485.

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

Liu, L.J., Zhang, Y.C., Li, Q.H., Sang, Y., Mao, J., Lian, H.L., Wang, L., and Yang, H.Q. (2008). COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in Arabidopsis. Plant Cell **20,** 292-306.

Lopez-Juez, E., Dillon, E., Magyar, Z., Khan, S., Hazeldine, S., de Jager, S.M., Murray, J.A., Beemster, G.T., Bogre, L., and Shanahan, H. (2008). Distinct light-initiated gene expression and cell cycle programs in the shoot apex and cotyledons of Arabidopsis. Plant Cell **20,** 947-968.

Lu, P., Porat, R., Nadeau, J.A., and O'Neill, S.D. (1996). Identification of a meristem L1 layer-specific gene in Arabidopsis that is expressed during embryonic pattern formation and defines a new class of homeobox genes. Plant Cell **8,** 2155-2168.

Lucas, W.J., Bouche-Pillon, S., Jackson, D.P., Nguyen, L., Baker, L., Ding, B., and Hake, S. (1995). Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Science **270,** 1980-1983.

Ma, L., Gao, Y., Qu, L., Chen, Z., Li, J., Zhao, H., and Deng, X.W. (2002). Genomic evidence for COP1 as a repressor of light-regulated gene expression and development in Arabidopsis. Plant Cell **14,** 2383-2398.

Martinez-Garcia, J.F., Huq, E., and Quail, P.H. (2000). Direct targeting of light signals to a promoter element-bound transcription factor. Science **288,** 859-863.

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

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

Mazzella, M.A., Bertero, D., and Casal, J.J. (2000). Temperature-dependent internode elongation in vegetative plants of *Arabidopsis thaliana* lacking phytochrome B and cryptochrome 1. Planta **210,** 497-501.

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

McCormac, A.C., and Terry, M.J. (2002). Light-signalling pathways leading to the co-ordinated expression of HEMA1 and Lhcb during chloroplast development in *Arabidopsis thaliana*. Plant J **32,** 549-559.

McDowell, J.M., An, Y.Q., Huang, S., McKinney, E.C., and Meagher, R.B. (1996). The arabidopsis *ACT7* actin gene is expressed in rapidly developing tissues and responds to several external stimuli. Plant Physiol **111,** 699-711.

McNellis, T.W., and Deng, X.W. (1995). Light control of seedling morphogenetic pattern. Plant Cell **7,** 1749-1761.

McNellis, T.W., von Arnim, A.G., Araki, T., Komeda, Y., Misera, S., and Deng, X.W. (1994). Genetic and molecular analysis of an allelic series of *cop1* mutants suggests functional roles for the multiple protein domains. Plant Cell **6,** 487-500.

Mittmann, F., Brucker, G., Zeidler, M., Repp, A., Abts, T., Hartmann, E., and Hughes, J. (2004). Targeted knockout in Physcomitrella reveals direct actions of phytochrome in the cytoplasm. Proc Natl Acad Sci U S A **101,** 13939-13944.

Miyawaki, K., Matsumoto-Kitano, M., and Kakimoto, T. (2004). Expression of cytokinin biosynthetic *isopentenyltransferase* genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J **37,** 128-138.

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

Mockler, T.C., Guo, H., Yang, H., Duong, H., and Lin, C. (1999). Antagonistic actions of Arabidopsis cryptochromes and phytochrome B in the regulation of floral induction. Development **126,** 2073-2082.

Moglich, A., Yang, X., Ayers, R.A., and Moffat, K. (2010). Structure and function of plant photoreceptors. Annu Rev Plant Biol **61,** 21-47.

Mondragon-Palomino, M., Hiese, L., Harter, A., Koch, M.A., and Theissen, G. (2009). Positive selection and ancient duplications in the evolution of class B floral homeotic genes of orchids and grasses. BMC Evol Biol **9,** 81.

Montgomery, B.L. (2008). Right place, right time: Spatiotemporal light regulation of plant growth and development. Plant Signal Behav **3,** 1053-1060.

Morelli, G., and Ruberti, I. (2000). Shade avoidance responses. Driving auxin along lateral routes. Plant Physiol **122,** 621-626.

Nagatani, A. (2004). Light-regulated nuclear localization of phytochromes. Curr Opin Plant Biol **7,** 708-711.

Nagatani, A., Chory, J., and Furuya, M. (1991). Phytochrome B is not detectable in the *hy3* mutant of Arabidopsis, which is deficient in responding to end-of-day far-red light treatments. Plant and Cell Physiology **32,** 1112-1119.

Nagatani, A., Reed, J.W., and Chory, J. (1993). Isolation and Initial Characterization of Arabidopsis Mutants That Are Deficient in Phytochrome A. Plant Physiol **102,** 269-277.

Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M., and Reed, J.W. (2000). *AXR2* encodes a member of the Aux/IAA protein family. Plant Physiol **123,** 563-574.

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

Neff, M.M., Fankhauser, C., and Chory, J. (2000). Light: an indicator of time and place. Genes Dev **14,** 257-271.

Newsham, K.K. (2003). UV-B radiation arising from stratospheric ozone depletion influences the pigmentation of the Antarctic moss *Andreaea regularis*. Oecologia **135,** 327-331.

Ni, M., Tepperman, J.M., and Quail, P.H. (1999). Binding of phytochrome B to its nuclear signalling partner PIF3 is reversibly induced by light. Nature **400,** 781-784.

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

Onodera, A., Kong, S.G., Doi, M., Shimazaki, K., Christie, J., Mochizuki, N., and Nagatani, A. (2005). Phototropin from *Chlamydomonas reinhardtii* is functional in *Arabidopsis thaliana*. Plant Cell Physiol **46,** 367-374.

Oravecz, A., Baumann, A., Mate, Z., Brzezinska, A., Molinier, J., Oakeley, E.J., Adam, E., Schafer, E., Nagy, F., and Ulm, R. (2006). *CONSTITUTIVELY PHOTOMORPHOGENIC1* is required for the UV-B response in Arabidopsis. Plant Cell **18,** 1975-1990.

Osterlund, M.T., Ang, L.H., and Deng, X.W. (1999). The role of COP1 in repression of Arabidopsis photomorphogenic development. Trends Cell Biol **9,** 113-118.

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

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

Ouellet, F., Overvoorde, P.J., and Theologis, A. (2001). IAA17/AXR3: biochemical insight into an auxin mutant phenotype. Plant Cell **13,** 829-841.

Park, E., Kim, J., Lee, Y., Shin, J., Oh, E., Chung, W.I., Liu, J.R., and Choi, G. (2004). Degradation of phytochrome interacting factor 3 in phytochrome-mediated light signaling. Plant Cell Physiol **45,** 968-975.

Parks, B.M., Hoecker, U., and Spalding, E.P. (2001). Light-induced growth promotion by SPA1 counteracts phytochrome-mediated growth inhibition during de-etiolation. Plant Physiol **126,** 1291-1298.

Pien, S., Wyrzykowska, J., McQueen-Mason, S., Smart, C., and Fleming, A. (2001). Local expression of expansin induces the entire process of leaf development and modifies leaf shape. Proc Natl Acad Sci U S A **98,** 11812-11817.

Powell, R.D.a.M., P.W. (1980). Opening of the hypocotyl hook in seedlings as influenced by light and adjacent tissues. Planta **1980,** 188-191.

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

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

Reed, J.W., and Chory, J. (1994). Mutational analyses of light-controlled seedling development in Arabidopsis. Semin Cell Biol **5,** 327-334.

Reed, J.W., Elumalai, R.P., and Chory, J. (1998). Suppressors of an *Arabidopsis thaliana phyB* mutation identify genes that control light signaling and hypocotyl elongation. Genetics **148,** 1295-1310.

Reed, J.W., Nagatani, A., Elich, T.D., Fagan, M., and Chory, J. (1994). Phytochrome A and Phytochrome B Have Overlapping but Distinct Functions in Arabidopsis Development. Plant Physiol **104,** 1139-1149.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M., and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. Plant Cell **5,** 147-157.

Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P.F., Lindquist, E.A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., Fujita, T., Oishi, K., Shin, I.T., Kuroki, Y., Toyoda, A., Suzuki, Y., Hashimoto, S., Yamaguchi, K., Sugano, S., Kohara, Y., Fujiyama, A., Anterola, A., Aoki, S., Ashton, N., Barbazuk, W.B., Barker, E., Bennetzen, J.L., Blankenship, R., Cho, S.H., Dutcher, S.K., Estelle, M., Fawcett, J.A., Gundlach, H., Hanada, K., Heyl, A., Hicks, K.A., Hughes, J., Lohr, M., Mayer, K., Melkozernov, A., Murata, T., Nelson, D.R., Pils, B., Prigge, M., Reiss, B., Renner, T., Rombauts, S., Rushton, P.J., Sanderfoot, A., Schween, G., Shiu, S.H., Stueber, K., Theodoulou, F.L., Tu, H., Van de Peer, Y., Verrier, P.J., Waters, E., Wood, A., Yang, L., Cove, D., Cuming, A.C., Hasebe, M., Lucas, S., Mishler, B.D., Reski, R., Grigoriev, I.V., Quatrano, R.S., and Boore, J.L. (2008). The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. Science **319,** 64-69.

Rensing, S.A., Rombauts, S., Van de Peer, Y., and Reski, R. (2002). Moss transcriptome and beyond. Trends Plant Sci **7,** 535-538.

Richardt, S., Lang, D., Reski, R., Frank, W., and Rensing, S.A. (2007). PlanTAPDB, a phylogeny-based resource of plant transcription-associated proteins. Plant Physiol **143,** 1452-1466.

Robson, F., Costa, M.M., Hepworth, S.R., Vizir, I., Pineiro, M., Reeves, P.H., Putterill, J., and Coupland, G. (2001). Functional importance of conserved domains in the flowering-time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. Plant J **28,** 619-631.

Rodriguez-Falcon, M., Bou, J., and Prat, S. (2006). Seasonal control of tuberization in potato: conserved elements with the flowering response. Annu Rev Plant Biol **57,** 151-180.

Rupprecht, L. (2010). Analysis of regulation of root growth through the COP1/SPA complex. Bachelor's Thesis, University of Cologne.

Saibo, N.J., Vriezen, W.H., Beemster, G.T., and Van Der Straeten, D. (2003). Growth and stomata development of Arabidopsis hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. Plant J **33,** 989-1000.

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

Sakamoto, K., and Nagatani, A. (1996). Nuclear localization activity of phytochrome B. Plant J **10,** 859-868.

Salisbury, F.J., Hall, A., Grierson, C.S., and Halliday, K.J. (2007). Phytochrome coordinates Arabidopsis shoot and root development. Plant J **50,** 429-438.

Salter, M.G., Franklin, K.A., and Whitelam, G.C. (2003). Gating of the rapid shade-avoidance response by the circadian clock in plants. Nature **426,** 680-683.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288,** 1613-1616.

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

Sato, Y., Wada, M., and Kadota, A. (2003). Accumulation response of chloroplasts induced by mechanical stimulation in bryophyte cells. Planta **216,** 772-777.

Savaldi-Goldstein, S., Peto, C., and Chory, J. (2007). The epidermis both drives and restricts plant shoot growth. Nature **446,** 199-202.

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

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

Searle, I., and Coupland, G. (2004). Induction of flowering by seasonal changes in photoperiod. EMBO J **23,** 1217-1222.

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

Seo, H.S., Yang, J.Y., Ishikawa, M., Bolle, C., Ballesteros, M.L., and Chua, N.H. (2003). LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. Nature **423,** 995-999.

Serrano, G., Herrera-Palau, R., Romero, J.M., Serrano, A., Coupland, G., and Valverde, F. (2009). Chlamydomonas CONSTANS and the evolution of plant photoperiodic signaling. Curr Biol **19,** 359-368.

Sessa, G., Carabelli, M., Sassi, M., Ciolfi, A., Possenti, M., Mittempergher, F., Becker, J., Morelli, G., and Ruberti, I. (2005). A dynamic balance between gene activation and repression regulates the shade avoidance response in Arabidopsis. Genes Dev **19,** 2811-2815.

Sessions, A., Weigel, D., and Yanofsky, M.F. (1999). The Arabidopsis thaliana MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and young primordia. Plant J **20,** 259-263.

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

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

Shen, H., Moon, J., and Huq, E. (2005). PIF1 is regulated by light-mediated degradation through the ubiquitin-26S proteasome pathway to optimize photomorphogenesis of seedlings in Arabidopsis. Plant J **44,** 1023-1035.

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

Shen, Y., Khanna, R., Carle, C.M., and Quail, P.H. (2007). Phytochrome induces rapid PIF5 phosphorylation and degradation in response to red-light activation. Plant Physiol **145,** 1043-1051.

Smith, H. (2006). in Photomorphogenesis in Plants and Bacteria, Function and Signal Transduction Mechanisms, eds Schafer E, Nagy F (Springer, Dordrecht, The Netherlands), 3rd Ed **641-651**.

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

Stadler, R., and Sauer, N. (1996). The Arabidopsis thaliana *AtSUC2* gene is specifically expressed in companion cells. . Bot. Acta **109,** 299-306.

Subramanian, C., Kim, B.H., Lyssenko, N.N., Xu, X., Johnson, C.H., and von Arnim, A.G. (2004). The Arabidopsis repressor of light signaling, COP1, is regulated by nuclear exclusion: mutational analysis by bioluminescence resonance energy transfer. Proc Natl Acad Sci U S A **101,** 6798-6802.

Sugano, S.S., Shimada, T., Imai, Y., Okawa, K., Tamai, A., Mori, M., and Hara-Nishimura, I. (2010). Stomagen positively regulates stomatal density in Arabidopsis. Nature **463,** 241-244.

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

Susek, R.E., Ausubel, F.M., and Chory, J. (1993). Signal transduction mutants of Arabidopsis uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. Cell **74,** 787-799.

Takano, M., Inagaki, N., Xie, X., Kiyota, S., Baba-Kasai, A., Tanabata, T., and Shinomura, T. (2009). Phytochromes are the sole photoreceptors for perceiving red/far-red light in rice. Proc Natl Acad Sci U S A **106,** 14705-14710.

Takano, M., Inagaki, N., Xie, X., Yuzurihara, N., Hihara, F., Ishizuka, T., Yano, M., Nishimura, M., Miyao, A., Hirochika, H., and Shinomura, T. (2005). Distinct and cooperative functions of phytochromes A, B, and C in the control of deetiolation and flowering in rice. Plant Cell **17,** 3311-3325.

Tamaki, S., Matsuo, S., Wong, H.L., Yokoi, S., and Shimamoto, K. (2007). Hd3a protein is a mobile flowering signal in rice. Science **316,** 1033-1036.

Tanaka, S., Nakamura, S., Mochizuki, N., and Nagatani, A. (2002). Phytochrome in cotyledons regulates the expression of genes in the hypocotyl through auxin-dependent and -independent pathways. Plant Cell Physiol **43,** 1171-1181.

Tao, Y., Ferrer, J.L., Ljung, K., Pojer, F., Hong, F., Long, J.A., Li, L., Moreno, J.E., Bowman, M.E., Ivans, L.J., Cheng, Y., Lim, J., Zhao, Y., Ballare, C.L., Sandberg, G., Noel, J.P., and Chory, J. (2008). Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell **133,** 164-176.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M.K., Harper, R.M., Liscum, E., and Yamamoto, K.T. (2004). *MASSUGU2* encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. Plant Cell **16,** 379-393.

Tian, Q., Nagpal, P., and Reed, J.W. (2003). Regulation of Arabidopsis SHY2/IAA3 protein turnover. Plant J **36,** 643-651.

Tian, Q., Uhlir, N.J., and Reed, J.W. (2002). Arabidopsis SHY2/IAA3 inhibits auxin-regulated gene expression. Plant Cell **14,** 301-319.

Truernit, E., and Sauer, N. (1995). The promoter of the Arabidopsis thaliana *SUC2* sucrose-H+ symporter gene directs expression of beta-glucuronidase to the phloem: evidence for phloem loading and unloading by SUC2. Planta **196,** 564-570.

Tsuge, T., Inagaki, N., Yoshizumi, T., Shimada, H., Kawamoto, T., Matsuki, R., Yamamoto, N., and Matsui, M. (2001). Phytochrome-mediated control of *COP1* gene expression in rice plants. Mol Genet Genomics **265,** 43-50.

Turck, F., Fornara, F., and Coupland, G. (2008). Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol **59,** 573-594.

Uenaka, H., and Kadota, A. (2007). Functional analyses of the *Physcomitrella patens* phytochromes in regulating chloroplast avoidance movement. Plant J **51,** 1050-1061.

Uenaka, H., Wada, M., and Kadota, A. (2005). Four distinct photoreceptors contribute to light-induced side branch formation in the moss *Physcomitrella patens*. Planta **222,** 623-631.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science **303,** 1003-1006.

Vandenbussche, F., Verbelen, J.P., and Van Der Straeten, D. (2005). Of light and length: regulation of hypocotyl growth in Arabidopsis. Bioessays **27,** 275-284.

Von Arnim, A., and Deng, X.W. (1996). Light Control of Seedling Development. Annu Rev Plant Physiol Plant Mol Biol **47,** 215-243.

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

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

Wada, M. and Kadota, A. (1989). Photomorphogenesis in lower plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40,** 169-191.

Wang, H., Kang, D., Deng, X.W., and Wei, N. (1999). Evidence for functional conservation of a mammalian homologue of the light-responsive plant protein COP1. Curr Biol **9,** 711-714.

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

Warnasooriya, S.N., and Montgomery, B.L. (2009). Detection of spatial-specific phytochrome responses using targeted expression of biliverdin reductase in Arabidopsis. Plant Physiol **149,** 424-433.

Wei, N., and Deng, X.W. (1996). The role of the *COP/DET/FUS* genes in light control of arabidopsis seedling development. Plant Physiol **112,** 871-878.

Weinl, C., Marquardt, S., Kuijt, S.J., Nowack, M.K., Jakoby, M.J., Hulskamp, M., and Schnittger, A. (2005). Novel functions of plant cyclin-dependent kinase inhibitors, ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis. Plant Cell **17,** 1704-1722.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmulling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15,** 2532-2550.

Whitelam, G.C., and Devlin, P.F. (1997). Roles of different phytochromes in Arabidopsis photomorphogenesis. Plant, Cell and Environment **20,** 752-758.

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

Wolf, L., Rizzini, L., Stracke, R., Ulm, R., and Rensing, S.A. (2010). The molecular and physiological responses of *Physcomitrella patens* to ultraviolet-B radiation. Plant Physiol **153,** 1123-1134.

Wu, G., and Spalding, E.P. (2007). Separate functions for nuclear and cytoplasmic cryptochrome 1 during photomorphogenesis of Arabidopsis seedlings. Proc Natl Acad Sci U S A **104,** 18813-18818.

Yamamoto, Y.T., Taylor, C.G., Acedo, G.N., Cheng, C.L., and Conkling, M.A. (1991). Characterization of cis-acting sequences regulating root-specific gene expression in tobacco. Plant Cell **3,** 371-382.

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

Yang, J., Lin, R., Sullivan, J., Hoecker, U., Liu, B., Xu, L., Deng, X.W., and Wang, H. (2005). Light regulates COP1-mediated degradation of HFR1, a transcription factor essential for light signaling in Arabidopsis. Plant Cell **17,** 804-821.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., and Sasaki, T. (2000). *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene *CONSTANS*. Plant Cell **12,** 2473-2484.

Yi, C., and Deng, X.W. (2005). COP1 - from plant photomorphogenesis to mammalian tumorigenesis. Trends Cell Biol **15,** 618-625.

Yoo, S.K., Chung, K.S., Kim, J., Lee, J.H., Hong, S.M., Yoo, S.J., Yoo, S.Y., Lee, J.S., and Ahn, J.H. (2005). CONSTANS activates SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 through FLOWERING LOCUS T to promote flowering in Arabidopsis. Plant Physiol **139,** 770-778.

Yu, J.W., Rubio, V., Lee, N.Y., Bai, S., Lee, S.Y., Kim, S.S., Liu, L., Zhang, Y., Irigoyen, M.L., Sullivan, J.A., Lee, I., Xie, Q., Paek, N.C., and Deng, X.W. (2008). COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability. Mol Cell **32,** 617-630.

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

Zeevaart, J.A.D. (1976). Physiology of flower formation. Annu Rev Plant Physiol. **27,** 321-348.

Zhang, Y.C., Gong, S.F., Li, Q.H., Sang, Y., and Yang, H.Q. (2006). Functional and signaling mechanism analysis of rice CRYPTOCHROME 1. Plant J **46,** 971-983.

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

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