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The *Arabidopsis* repressor of light signaling SPA1 acts in the phloem to regulate seedling de-etiolation, leaf expansion and flowering time

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SUMMARY

Plants adjust their growth and development in response to the ambient light environment. These light responses involve systemic signals that coordinate differentiation of different tissues and organs. Here, we have investigated the function of the key repressor of photomorphogenesis SPA1 in different tissues of the plant by expressing GUS-SPA1 under the control of tissue-specific promoters in a *spa* mutant background. We show that SPA1 expression in the phloem vasculature is sufficient to rescue the *spa1* mutant phenotype in dark-grown *spa* mutant seedlings. Expression of SPA1 in mesophyll, epidermis or root tissues of the seedling, by contrast, has no or only slight effects. In the leaf, SPA1 expression in both the phloem and the mesophyll is required for full complementation of the defect in leaf expansion. SPA1 in phloem and mesophyll tissues affected division and expansion of cells in the epidermal layer, indicating that SPA1 induces non-cell-autonomous responses also in the leaf. Photoperiodic flowering is exclusively controlled by SPA1 expression in the phloem, which is consistent with previous results showing that the direct substrate of the COP1/SPA complex, CONSTANS, also acts in the phloem. Taken together, our results highlight the importance of phloem vascular tissue in coordinating growth and development. Because the SPA1 protein itself is incapable of moving from cell to cell, we suggest that SPA1 regulates the activity of downstream component(s) of light signaling that subsequently act in a non-cell-autonomous manner. SPA1 action in the phloem may also result in mechanical stimuli that affect cell elongation and cell division in other tissues.

KEY WORDS: SPA1, Cell-cell communication, Photomorphogenesis, Stomata differentiation, Flowering time, Leaf expansion, *Arabidopsis*

INTRODUCTION

Light is an important informational cue with which to regulate many stages of plant growth and development. To sense the ambient light conditions, plants have evolved multiple photoreceptors, which include the red/far-red light (R/FR)-sensing phytochromes and the UV-A/blue light-sensing cryptochromes and phototropins (Chen et al., 2004; Whitelam and Halliday, 2007). Activated photoreceptors initiate at least two signaling cascades in *Arabidopsis*. First, phytochromes in their active conformation interact with PHYTOCHROME INTERACTING FACTORS (PIF proteins), most of which are subsequently phosphorylated and degraded in the proteasome. PIF proteins activate the normal dark response of seedlings and their degradation is, therefore, necessary for photomorphogenesis (Leivar and Quail, 2010). Second, phytochromes and cryptochromes are thought to inhibit the COP1/SPA complex, which acts as an E3 ubiquitin ligase in darkness to ubiquitylate transcription factors required for light signaling (Yi and Deng, 2005). The COP1/SPA complex consists of two essential components, COP1 and members of the four-member SPA protein family, which act in concert with the CULLIN4-DDB1 complex (Chen et al., 2010; Laubinger et al., 2004; Zhu et al., 2008). *cop1* and *spa1 spa2 spa3 spa4* quadruple mutants fail to degrade the substrate transcription factors and, therefore, show features of light-grown seedlings even when grown in complete darkness (Kang et al., 2009; Laubinger et al., 2004; Osterlund et al., 1999; Zhu et al., 2008).

Though primarily active in darkness, the COP1/SPA complex is also important for fine-tuning de-etiolation of light-grown seedlings because *spa1*, *spa3* and *spa4* mutant seedlings show exaggerated photomorphogenesis in the light (Hoecker et al., 1998; Laubinger and Hoecker, 2003). Furthermore, *cop1* and *spa1 spa2 spa3 spa4* mutant leaves are much smaller than wild-type leaves (Deng and Quail, 1992; Laubinger et al., 2004). *cop1* and *spa* mutants also fail to delay flowering in short days (Laubinger et al., 2004; McNellis et al., 1994). The COP1/SPA complex controls photoperiodic flowering by regulating the stability of the floral inducer CONSTANS (Jang et al., 2008; Laubinger et al., 2006; Liu et al., 2008).

The four SPA genes exhibit redundant but also distinct functions throughout plant development. Nevertheless, the *SPA1* gene provides the most significant contribution among the four SPA genes. *SPA1* is important for seedling growth in the light and in darkness, leaf expansion and photoperiodic flowering (Fittinghoff et al., 2006; Laubinger et al., 2004; Laubinger et al., 2006).

Light initiates developmental responses through non-cell-autonomous signaling (Bou-Torrent et al., 2008; Montgomery, 2008). Here, the photoperiodic induction of flowering is the best-studied example. Day length is perceived by leaves, 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). Consistent with this, CO, the transcription factor that activates *FT* transcription, acts in the

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phloem (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). By contrast, phyB acts in mesophyll cells to inhibit the induction of flowering (Endo et al., 2005). Thus, non-cell-autonomous effects from both the phloem and the mesophyll operate to regulate photoperiodic flowering.

Micro-beam irradiation of cotyledons induces a light-responsive promoter also outside the irradiated areas, indicating intercellular communication in seedlings (Bischoff et al., 1997). In *Arabidopsis* seedlings, expression of PHYB in cotyledon mesophyll complemented the hypocotyl-length phenotype of the *phyB* mutant (Endo et al., 2005). Similarly, inhibiting phytochrome function specifically in cotyledon mesophyll resulted in elongation of the hypocotyl, indicating movement of a phytochrome-dependent signal from cotyledons to the hypocotyl (Warnasooriya and Montgomery, 2009). Auxin biosynthesis and polar auxin transport are required for the increase in hypocotyl elongation in response to low R/FR irradiation, suggesting that auxin contributes to inter-organ communication (Keuskamp et al., 2010; Tao et al., 2008). Consistent with this idea, the low R/FR signal is perceived by the leaf blade and led to increased elongation of the petiole via a process requiring polar auxin transport (Kozuka et al., 2010). However, polar auxin transport was not required for hypocotyl elongation in dark-grown seedlings (Jensen et al., 1998). By contrast, GA biosynthesis as well as normal cytokinin levels and auxin signaling are required for etiolation of seedlings in darkness (Alabadi et al., 2004; Reed et al., 1998).

Thus far, the functions of light signaling intermediates have not been investigated at tissue resolution. We therefore examined whether SPA1, as a member of the COP1/SPA complex, participates in or induces non-cell-autonomous signaling events.

MATERIALS AND METHODS

Plant material and growth conditions

spa1-7, *spa3-1 spa4-1* and *spa1-7 spa3-1 spa4-1* (Col), and *spa1-2*, *phyB-1*, *spa1-2 phyB-1* (RLD) have been described previously (Fittinghoff et al., 2006; Hoecker et al., 1998; Parks et al., 2001). *spa1-7 spa2-1 spa3-1* and *spa1-7 cry2-1* (Guo et al., 1998) were generated by crossing and confirmed using polymorphic markers and cry2 immunoblotting.

LED light sources, growth conditions and phenotype analyses have been described previously (Kang et al., 2009; Laubinger et al., 2004; Laubinger et al., 2006).

Plasmid construction and genetic analysis of transgenic plants

To generate a GUS-SPA1 destination vector, polylinkers (*Nco*I-*Apa*I-*Sac*I-*Nco*I and *Sal*I-*Sac*I-*Not*I-*Sal*I) were inserted into the *Nco*I and *Sal*I sites of pRTL2/GUS-SPA1 (Hoecker et al., 1999). GUS-SPA1 was subsequently excised by partial *Sac*I digestion and ligated into the *Sac*I site of pGWB1 (Nakagawa et al., 2007). The 2260 bp *SPA1* promoter, the 1537 bp *CAB3* promoter and the 1208 bp *CER6* promoter (upstream of the respective coding region) were PCR amplified from Columbia genomic DNA and subsequently introduced into the pDONR221 vector (Invitrogen). All other promoter entry clones have been described previously (An et al., 2004). *Promoter::GUS-SPA1* constructs were generated through LR reactions between the pGWB1/GUS-SPA1 and promoter entry clones and subsequently transformed into the *spa1 spa2 spa3* and the *spa1 spa3 spa4* mutants.

Determination of epidermis cell number and size

The fully expanded leaf three of 3-week-old plants was cleared in 95% ethanol followed by rehydration in a graded ethanol series. The average number of epidermis cells per leaf was calculated from the total leaf area, as determined by ImageJ (Bethesda, MA, USA), and the number of epidermis cells in a defined area of the leaf. Total leaf area and average number of epidermis cells in the leaf were used to calculate the average size of an epidermis cell. All error bars shown indicate the s.e.m.

Histochemical analysis of GUS activity

Whole tissues were vacuum-infiltrated in GUS-staining buffer [0.5 mM $NaPO_4$ (pH 7.0), 10 mM EDTA, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 1 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-Gluc) and 0.1% Triton X-100], incubated at 37°C and then destained in 70% ethanol.

Cross-sections of leaf and stem tissues were prepared using a razor blade followed by staining procedures as described above. For cross-sections of cotyledons, GUS-stained seedlings were fixed (50% ethanol, 5% acetic acid, 3% formaldehyde), dehydrated in a graded ethanol series (70%, 96% and 100%; 2 hours each), embedded in Technovit 7100 (Heraeus Kulzer) according to the manufacturer's instructions and sectioned using a microtome.

In situ hybridization

In situ hybridization followed the protocol of Jackson (Jackson, 1991). Paraffin-embedded tissue was sectioned using a Leica RM2145 rotary microtome (7-10 μm). *SPA1* probes were obtained from nucleotides +63 to +430 relative to the start codon of the open reading frame and fused to the T7 promoter in sense and antisense orientations. Digoxigenin-labeled RNA probes were obtained as described previously (Bradley et al., 1993).

RNA isolation and transcript analysis

RNA isolation and qRT-PCR were performed as described previously (Balcerowicz et al., 2011). Primers for *FT* and *UBQ10* amplification have been described previously (Endo et al., 2007; Balcerowicz et al., 2011).

RESULTS

Spatial pattern of SPA1 accumulation in *Arabidopsis*

We first conducted a *SPA1* RNA in situ analysis to determine which tissues express *SPA1*. *SPA1* mRNA accumulated ubiquitously in seedlings and leaves (Fig. 1A-J). *SPA1* transcript levels were particularly high in vascular tissue of dark-grown seedlings (Fig. 1C). In developing leaves, SPA1 mRNA levels were lower in epidermal tissue than in inner tissues (Fig. 1I). The spatial *SPA1* transcript accumulation was very similar in the *spa* mutant backgrounds used in the studies described below (see Fig. S1 in the supplementary material). This ubiquitous expression pattern was also observed in transgenic plants expressing a GUS-SPA1 fusion protein under the control of the native *SPA1* promoter (*SPA1::GUS-SPA1*) (Fig. 1K-Z). Here, we used two *spa* triple mutant backgrounds for transgene expression in order to be able to assess the functionality of the transgene throughout development, which is partly masked by redundancy among the four *SPA* genes. Transgenic lines showed full complementation of the *spa1 spa2 spa3* triple mutant seedling phenotype and the *spa1 spa3 spa4* mutant adult phenotype (leaf size and flowering time), respectively, indicating that the transgene was fully functional (Figs 3, 4, 8, 9, see also below). In seedlings, GUS activity was higher in cotyledons and the root tip than in the hypocotyl (Fig. 1K-O,Q-U). GUS-SPA1 accumulated in the epidermis, the mesophyll and – at particularly high levels – in vascular bundles (Fig. 1M,O,P,S,U,V). Also in adult plants, GUS-SPA1 accumulated ubiquitously (Fig. 1W-Z), with very strong expression in vascular bundles and lower expression in the epidermis (Fig. 1X,Y). In stem vasculature, GUS-SPA1 was primarily expressed in the phloem (Fig. 1Z).

Tissue-specific expression of GUS-SPA1 in transgenic *spa1 spa3 spa4* plants

Adult *spa1 spa3 spa4* mutants are very small in size and show extremely early flowering in short days when compared with wild-type plants (Laubinger et al., 2004; Laubinger et al., 2006). We therefore used this background to investigate in which tissues SPA1

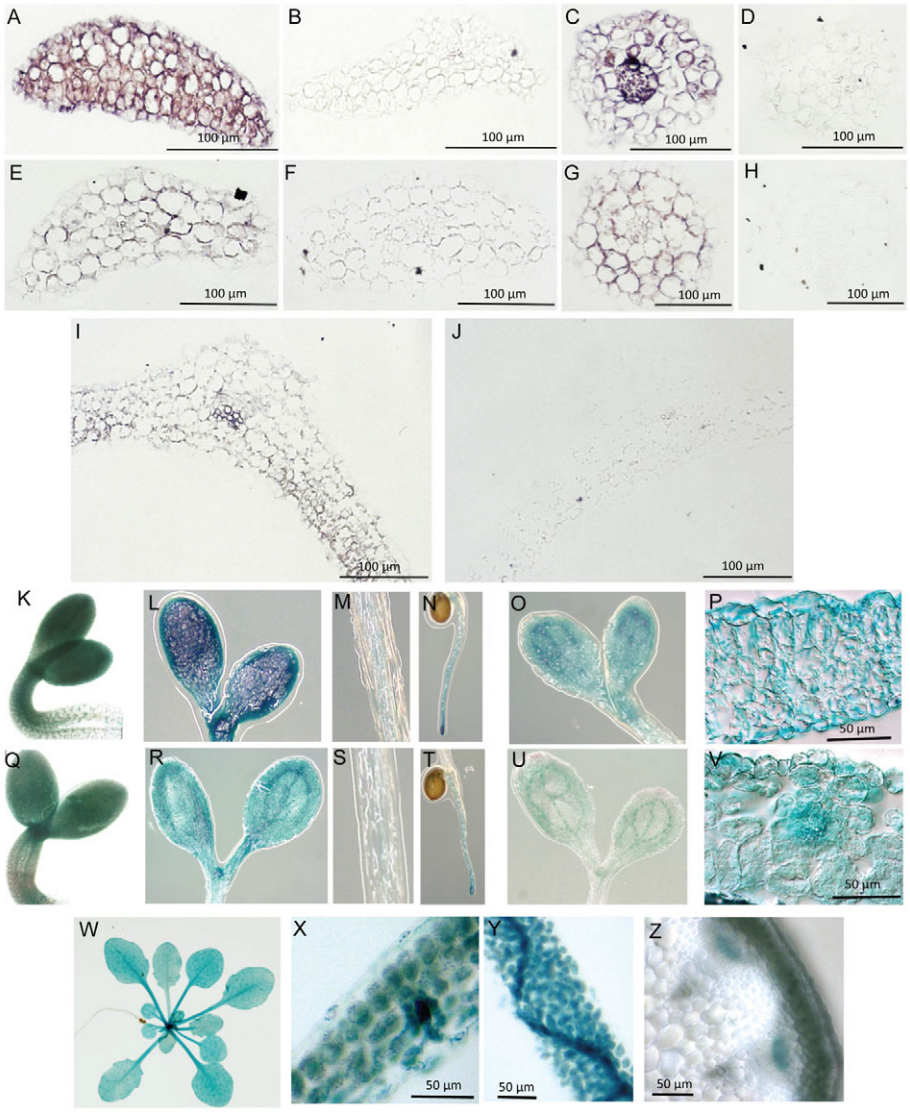

Fig. 1. *SPA1* is ubiquitously expressed in seedlings and adult plants of *Arabidopsis*. (**A-J**) In situ hybridization to *SPA1* mRNA in cross-sections of cotyledons and hypocotyls of dark-grown (A-D) and light-grown (E-H) wild-type seedlings and of leaves (I,J) of wild-type plants with antisense (A,C,E,G,I) and sense (B,D,F,H,J) probes. (**K-V**) GUS-SPA1 accumulation in dark-grown (K-P) and light-grown (Q-V) transgenic *spa1 spa2 spa3* seedlings expressing *GUS-SPA1* under the control of the native *SPA1* promoter. Seedling 36 hours after germination stained overnight for GUS activity (K,Q); cotyledons (L,R), hypocotyl (M,S) and root (N,T) of a 4-day-old seedling stained overnight for GUS activity; cotyledons of a 4-day-old seedling after 4 hours of GUS-staining (O,U); cross-section through a cotyledon stained overnight for GUS activity (P,V). (**W-Z**) GUS-SPA1 accumulation in transgenic *spa1 spa3 spa4* plants expressing *GUS-SPA1* under the control of the native *SPA1* promoter. A 3-week-old plant (W); Cross-sections through leaves (X,Y) and the inflorescence stem (Z). All tissues were stained overnight for GUS activity.

acts to regulate photoperiodic flowering and leaf expansion. To this end, a GUS-SPA1 fusion protein was expressed under the control of tissue-specific promoters. Promoters used were p*SUC2* for phloem-specificity (Stadler and Sauer, 1996; Truernit and Sauer, 1995), p*CAB3* for mesophyll-specificity (Susek et al., 1993), p*ML1* and p*CER6* for epidermis specificity (Hooker et al., 2002; Lu et al., 1996; Sessions et al., 1999), p*KNAT1* for shoot meristem specificity (Lincoln et al., 1994) and p*TobRB7* for root specificity (Yamamoto et al., 1991). Previously, these promoters have successfully been used to study photoperiodic flowering (An et al., 2004; Endo et al., 2007), seedling development (Endo et al., 2007; Savaldi-Goldstein et al., 2007; Warnasooriya and Montgomery, 2009) and other phenotypes such as shoot branching (Booker et al., 2003).

For each *promoter::GUS-SPA1* construct, at least 40 independent transgenic lines were analyzed for tissue-specific expression and complementation of the *spa* mutant phenotype. Among these, three representative lines were propagated to obtain homozygous transgenic lines that are presented in Fig. 2 and Figs S2, S3 in the supplementary material. When expressed from the *SUC2* promoter, GUS-SPA1 accumulated exclusively in vascular bundles (Fig. 2A-E and see Figs S2, S3 in the supplementary material). GUS activity was highest in developed leaves and weaker, but detectable, in very young leaves (~2 mm length). Leaf primordia, by contrast, did not show detectable GUS-SPA1 expression (data not shown). Such stage-dependent activity of the *SUC2* promoter has also been reported in previous studies (Imalu et al., 1999; Truernit and Sauer, 1995).

In *CAB3::GUS-SPA1* transgenic lines, GUS-SPA1 expression was restricted to mesophyll cells (Fig. 2F,G and see Figs S2, S3 in the supplementary material). Exclusive expression of GUS-SPA1 in the epidermis was detected in *ML1::GUS-SPA1* (Fig. 2H,I and see Figs S2, S3 in the supplementary material) and *CER6::GUS-SPA1* (Fig. 2J,K and see Figs S2, S3 in the supplementary material) lines. The expression levels using both promoters, *ML1* and *CER6*, were high at the shoot apex, in leaf primordia and in young developing leaves, and decreased to lower levels in developed leaves. *KNAT1::GUS-SPA1* and *TobRB7::GUS-SPA1* transgenic lines showed shoot meristem- and root-specific expression of GUS-SPA1, respectively (Fig. 2L,M and see Figs S2, S3 in the supplementary material). In all transgenic lines, the levels of GUS-SPA1 in the respective tissues were either similar to or higher than those of GUS-SPA1 when expressed under the control of the native *SPA1* promoter (Figs 1, 2).

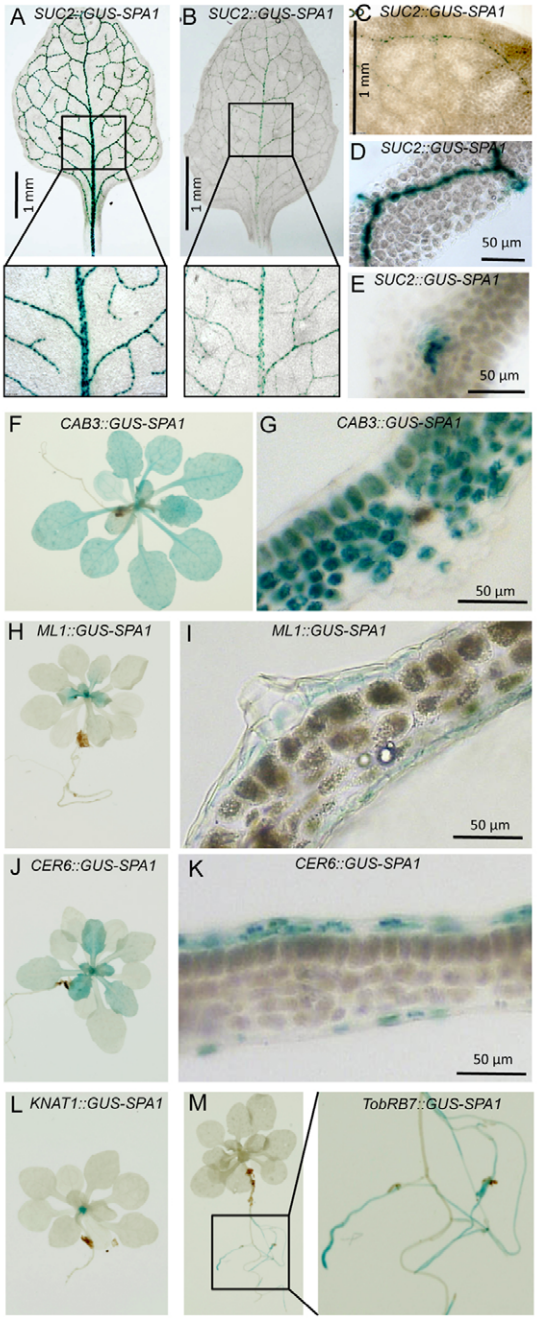

Fig. 2. GUS-SPA1 accumulation in transgenic *spa1 spa3 spa4* mutant plants expressing *GUS-SPA1* under the control of tissue-specific promoters. (**A-E**) Mature (A), developing (B) and very young (C) leaves, and cross-sections through leaves (D,E) of plants expressing *GUS-SPA1* from the *SUC2* promoter (line 11-5). (**F-M**) Whole plants and cross-sections through leaves of plants expressing *GUS-SPA1* from the promoters *CAB3* (F,G; line 1-1), *ML1* (H,I; line 15-10), *CER6* (J,K; line 1-6), *KNAT1* (L; line 1-3) and *TobRB7* (M; line 3-6). All tissues were stained overnight for GUS activity.

SPA1 acts in the phloem to regulate photoperiodic flowering

We examined in which tissues SPA1 acts to regulate flowering time. The *spa1 spa3 spa4* triple mutant used for these experiments flowers very early in short days and slightly early in long days when compared with the wild type (Fig. 3 and see Fig. S4A in the supplementary material) (Laubinger et al., 2006). Expression of *GUS-SPA1* under the control of the native *SPA1* promoter caused full complementation of the early-flowering phenotype of the *spa1 spa3 spa4* triple mutant (Fig. 3; see Fig. S4A in the supplementary material). Similarly, *SUC2::GUS-SPA1* transgenic lines, which express GUS-SPA1 exclusively in the phloem, showed full complementation of the early-flowering phenotype of the parental *spa* triple mutant. By contrast, expression of *GUS-SPA1* in any other tissue failed to rescue the *spa* triple mutant phenotype (Fig. 3 and see Fig. S4A in the supplementary material). In summary, these data demonstrate that SPA1 expression in the phloem is sufficient to inhibit flowering. Consistent with this finding, phloem-specific expression of GUS-SPA1 reduced *FT* transcript levels (see Fig. S5 in the supplementary material).

SPA1 acts in phloem and mesophyll tissues to regulate leaf expansion

The *spa3 spa4* double mutant, with functional *SPA1*, has larger leaves than the *spa1 spa3 spa4* triple mutant, indicating that *SPA1* plays a significant role in leaf expansion (Fittinghoff et al., 2006; Laubinger et al., 2004) (Fig. 4A,B). We therefore analyzed this phenotype in transgenic *spa1 spa3 spa4* plants expressing *promoter::GUS-SPA1* constructs. As expected, transgenic *SPA1::GUS-SPA1* lines showed full complementation of the leaf-size phenotype of the parental *spa* triple mutant in short and long days (Fig. 4A,B; see Fig. S4B in the supplementary material). Phloem-specific as well as mesophyll-specific expression of *GUS-SPA1* under the control of the *SUC2* and *CAB3* promoters, respectively, partially complemented the leaf-size phenotype of the *spa* triple mutant. By contrast, expression of *GUS-SPA1* in the epidermis (*ML1*, *CER6*), the shoot meristem (*KNAT1*) or the root (*TobRB7*) failed to complement the *spa* mutant leaf-size phenotype. These data indicate that SPA1 acts in both the phloem and the leaf mesophyll to regulate leaf expansion.

Because leaves of the *spa1 spa3 spa4* mutant contain fewer and smaller epidermal cells when compared with the wild type (P.F. and U.H., unpublished), we investigated whether expression of GUS-SPA1 rescued the cell division and/or cell expansion defect of epidermal cells. Fig. 4C,D shows that GUS-SPA1 expression in phloem or mesophyll tissues led to an increase in the number and, more weakly, the size of leaf epidermal cells. By contrast, epidermis-specific expression of GUS-SPA1 did not affect the number or size of epidermal cells. These results show that SPA1 activity in phloem and mesophyll tissues regulates division and expansion of epidermal cells. This indicates that SPA1 influences the epidermis through non-cell-autonomous effects.

None of the tissue-specific promoters used was capable of fully rescuing the *spa1* mutant leaf phenotype. This suggests that SPA1 expression in more than one tissue is necessary for full SPA1 function in the leaf. To test this possibility, we expressed SPA1 in both phloem and mesophyll tissues by crossing transgenic plants harboring the *CAB3::GUS-SPA1* construct with those carrying the *SUC2::GUS-SPA1* construct. Indeed, these double transgenic plants exhibited full complementation of the leaf-size phenotype of the parental *spa* triple mutant (Fig. 5A,B).

Genetic interaction of *SPA1* and photoreceptors in controlling photoperiodic flowering and leaf expansion

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

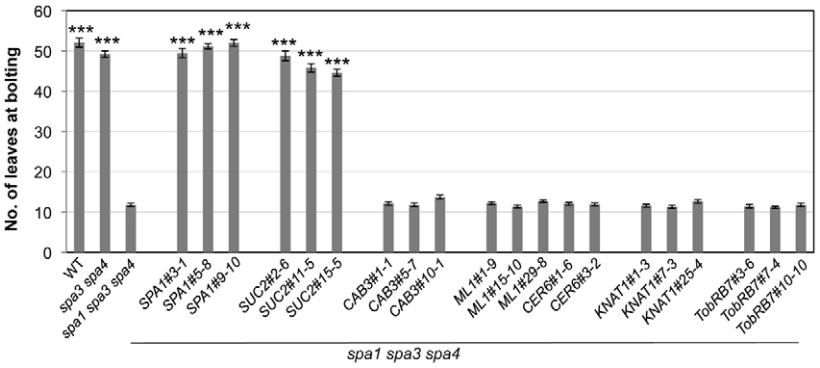

Fig. 3. SPA1 acts in the phloem to regulate flowering time. Flowering time of short-day-grown transgenic plants expressing GUS-SPA1 in specific tissues of the *spa1 spa3 spa4* mutant. For each transgene, several independent homozygous transgenic lines were analyzed, as indicated by the line numbers. Asterisks indicate significant differences between the indicated genotypes and the *spa1 spa3 spa4* mutant (*t*-test, $P<0.001$). Data are mean±s.e.m.

regulate flowering time (Endo et al., 2007; Endo et al., 2005). Additionally, phyB has been suggested to act in the mesophyll to regulate rosette leaf morphology (Endo et al., 2005). We therefore investigated the epistatic relationship between SPA1 action and photoreceptor activity.

The *spa1 cry2* double mutant flowered as early as the *spa1* single mutant, indicating that *spa1* is fully epistatic to *cry2* (Fig. 6A,B). This indicates that *SPA1* acts genetically downstream of *CRY2* in the same pathway. This result is consistent with the model that cry2 promotes flowering by inhibiting COP1/SPA ubiquitin

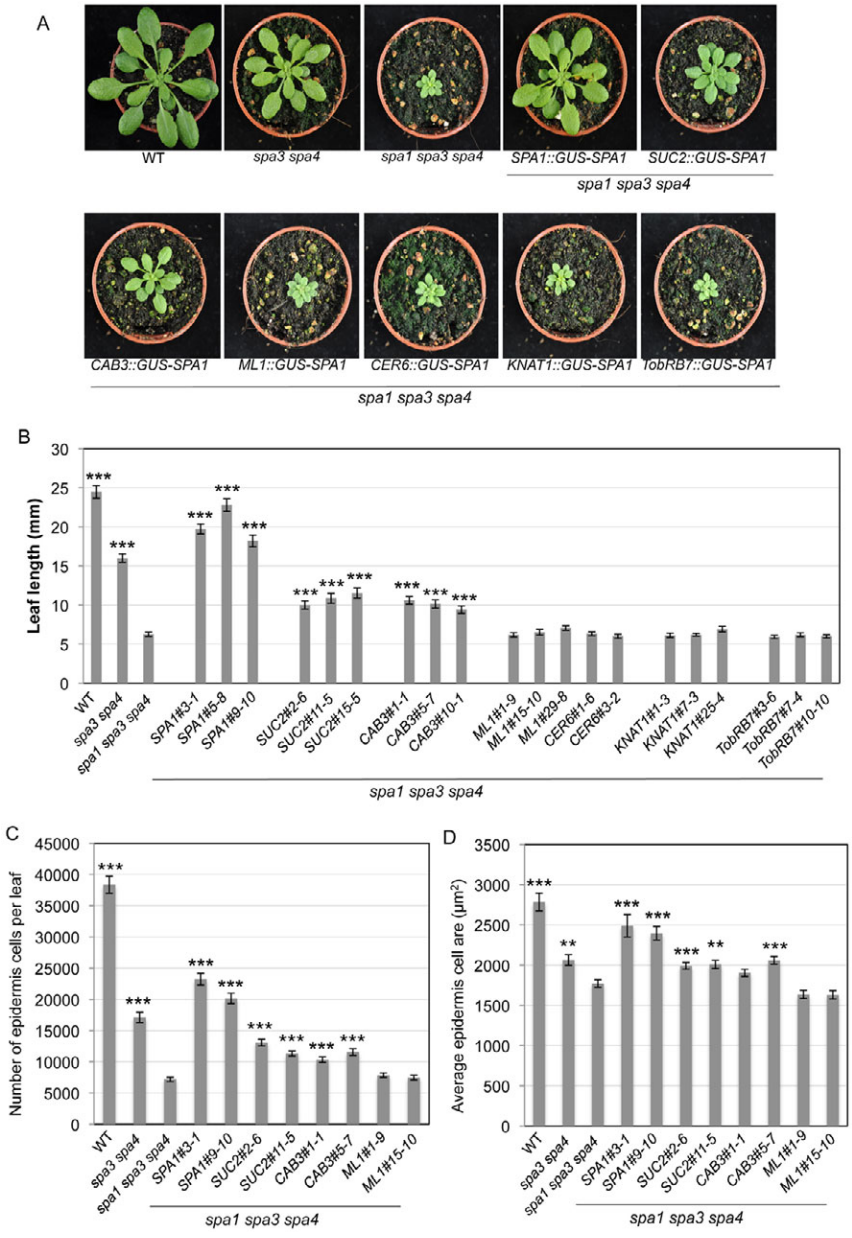

Fig. 4. SPA1 acts in the phloem and the mesophyll to regulate leaf size. (**A**) Visual phenotype of 4-week-old short-day-grown transgenic *spa1 spa3 spa4* mutant plants expressing GUS-SPA1 under the control of tissue-specific promoters, as indicated. As controls, wild-type (WT), *spa3 spa4* and *spa1 spa3 spa4* mutant plants are shown. (**B**) Quantification of leaf length of the genotypes shown in A. Several independent homozygous transgenic lines were analyzed for each transgene. (**C**) Average number of epidermis cells in leaf 3 of 3-week-old plants. Genotypes were as in A. (**D**) Average size of the epidermal cells in leaf 3 of the genotypes shown in A. Asterisks indicate significant differences between the indicated genotypes and the *spa1 spa3 spa4* mutant (*t*-test, $**P<0.01$, $***P<0.001$). Data are mean±s.e.m.

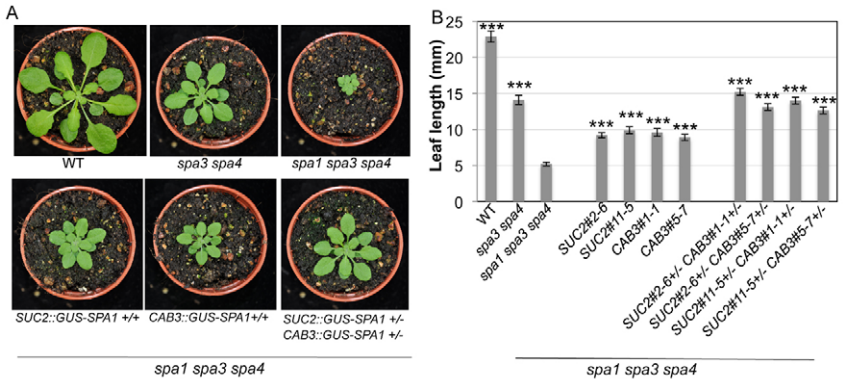

Fig. 5. SPA1 activities in the phloem and the mesophyll have additive effects on leaf size. (**A**) Visual phenotype of transgenic *SUC2::GUS-SPA1* (line 2-6), *CAB3::GUS-SPA1* (line 1-1) plants and an F1 plant that is hemizygous for both transgenes. As controls, wild-type (WT), *spa3 spa4* and *spa1 spa3 spa4* mutant plants are shown. All plants were 4 weeks old and grown in short day. (**B**) Quantification of leaf length of the genotypes shown in A. Four double transgenic lines were derived from respective crosses of two independent homozygous transgenic lines carrying *SUC2::GUS-SPA1* or *CAB3::GUS-SPA1*. Asterisks indicate significant differences between the indicated genotypes and the *spa1 spa3 spa4* mutant (*t*-test, $P<0.001$). Data are mean±s.e.m.

ligase activity (Liu et al., 2008). *spa1* and *phyB* single mutants flowered early in short days when compared with wild-type plants (Fig. 6C). The *spa1 phyB* double mutant flowered even earlier than both single mutants, indicating that *spa1* and *phyB* mutations act additively. This suggests that *SPA1* and *PHYB* act in independent pathways to regulate photoperiodic flowering.

Leaves of the *phyB* mutant show constitutive shade avoidance, displaying smaller leaf blades and longer petioles when compared with leaves of wild-type plants (Reed et al., 1993) (Fig. 6D). By contrast, leaves of the *spa1 phyB* double mutant did not exhibit a constitutive shade avoidance response. 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, here *SPA1* acts downstream of *PHYB* in the same pathway.

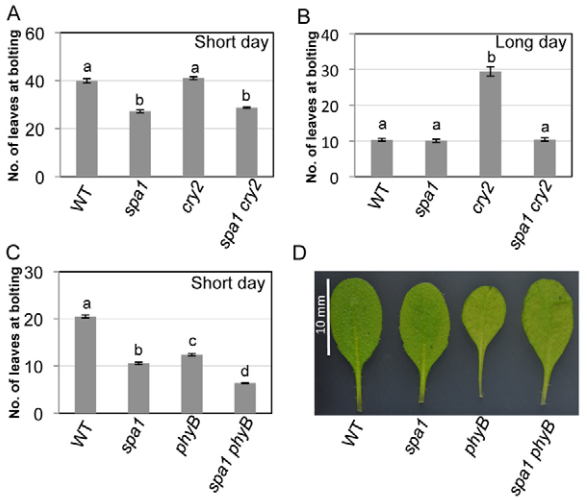

Fig. 6. Genetic interaction of *spa1* with *cry2* and *phyB* mutations. (**A**,**B**) Flowering time of wild-type (WT, Col-0), *spa1-7*, *cry2* and *spa1 cry2* double mutant plants grown in short days (A) and long days (B). (**C**) Flowering time of wild-type (WT, RLD), *spa1-2*, *phyB* and *spa1 phyB* double mutant plants grown in short days. (**D**) Leaf morphology of wild-type (WT, RLD), *spa1-2*, *phyB* and *spa1 phyB* double mutant plants grown in short days for 3 weeks. Lines designated with the same letter exhibit no significant difference in flowering time (*t*-test, $P<0.001$). Data are mean±s.e.m.

Tissue-specific expression of GUS-SPA1 in transgenic *spa1 spa2 spa3* seedlings

To express GUS-SPA1 in specific tissues of the seedling, the *spa1 spa2 spa3* mutant background was used. This mutant shows constitutive photomorphogenesis in darkness, a phenotype that is not observed in the *spa1 spa3 spa4* mutant used for the analysis of adult phenotypes (Laubinger et al., 2004). We employed the same tissue-specific promoters as used for studying flowering time and leaf expansion, except for the *CER6* promoter, which is not expressed in dark-grown seedlings (Hooker et al., 2002). Additionally, p*RolC* was used as a phloem-specific promoter (Booker et al., 2003) in dark-grown seedlings because we found that p*SUC2* failed to confer true phloem-specific expression in cotyledons of dark-grown seedlings that were more than 2 days old (data not shown).

Again, three representative transgenic lines were propagated to obtain homozygous lines which are shown in Fig. 7 and Figs S6, S7 in the supplementary material. The *RolC* promoter conferred vascular-specific expression of GUS-SPA1 in both dark- and light-grown seedlings. We analyzed 4-day-old seedlings that had fully germinated (Fig. 7B,D; see Figs S6, S7 in the supplementary material), as well as seedlings that had just cracked the seed coat and thus had not yet initiated hypocotyl elongation (36 hours after the induction of germination; Fig. 7A,C; see Figs S6, S7 in the supplementary material). At both stages, GUS activity was limited to the vasculature. In freshly imbibed seeds, by contrast, no GUS activity was detectable (data not shown). Transgenic *SUC2::GUS-SPA1* lines also accumulated GUS specifically in the vasculature of light-grown seedlings (Fig. 7Q,R; see Figs S6, S7 in the supplementary material). The *CAB3* and *ML1* promoters conferred GUS-SPA1 expression specifically in the mesophyll and epidermis, respectively (Fig. 7G-N; see Figs S6, S7 in the supplementary material). *KNAT1::GUS-SPA1* and *TobRB7::GUS-SPA1* transgenic lines showed shoot meristem- and root-specific expression of GUS-SPA1, respectively (Fig. 7E,F,O,P; see Figs S6, S7 in the supplementary material). Most tissue-specific promoters conferred similar levels of GUS-SPA1 accumulation in the respective tissues

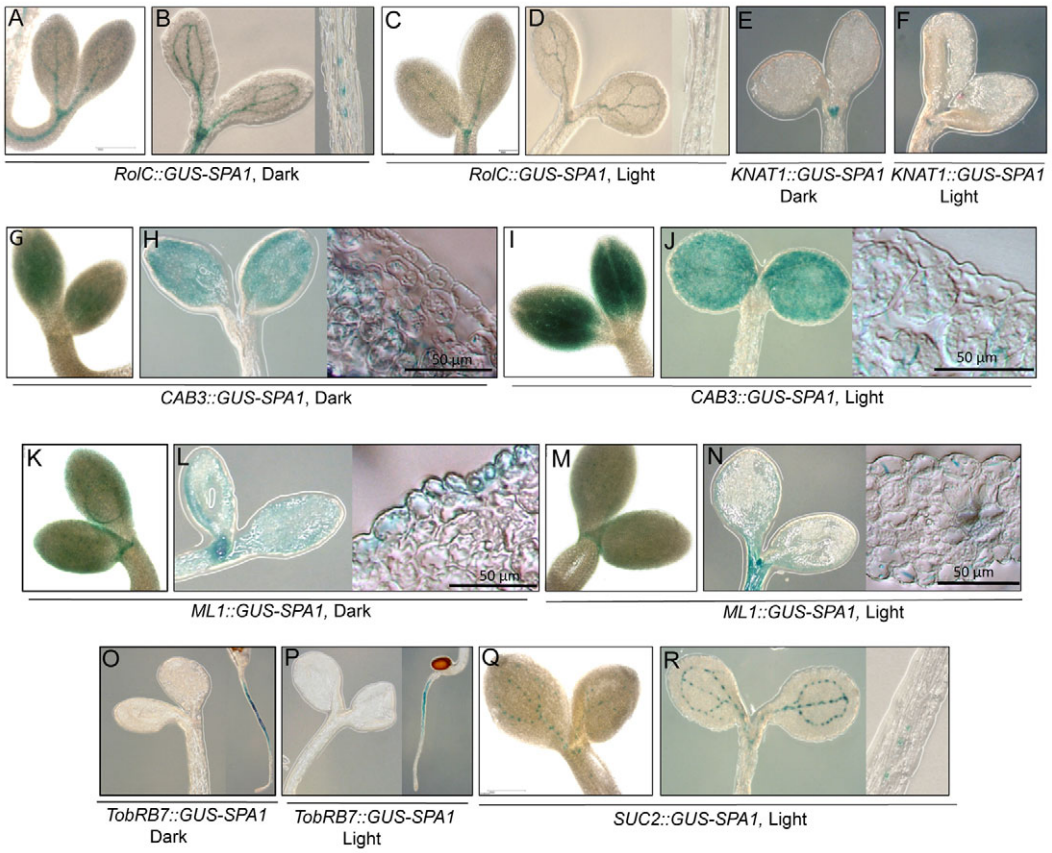

Fig. 7. GUS-SPA1 accumulation in transgenic *spa1 spa2 spa3* mutant seedlings expressing *GUS-SPA1* under the control of tissue-specific promoters. (**A-D**) *RolC::GUS-SPA1* (line 50-10). GUS staining of dark-grown (A,B) and light-grown (C,D) seedlings 36 hours (A,C) or 4 days (B,D) after the induction of germination. B and D show cotyledons and hypocotyl. Seedlings in A and C were manually removed from the seed coat. (**E**,**F**) *KNAT1::GUS-SPA1* (line 50-6). Four-day-old dark-grown (E) and light-grown (F) seedlings. (**G-J**) *CAB3::GUS-SPA1* (line 21-7). Dark-grown (G,H) and light-grown (I,J) seedlings 36 hours (G,I) or 4 days (H,J) after the induction of germination. H and I show cotyledons and cross-sections through cotyledons. (**K-N**) *ML1::GUS-SPA1*(line 6-1). Dark-grown (K,L) and light-grown (M,N) seedlings 36 hours (K,M) or 4 days (L,N) after the induction of germination. L and N show cotyledons and cross-sections through cotyledons. (**O**,**P**) *TobRB7::GUS-SPA1* (line 1-2). Cotyledons, hypocotyls and roots of 4-day-old dark-grown (O) and light-grown (P) seedlings. (**Q**,**R**) *SUC2::GUS-SPA1* (line 8-6). Light-grown seedlings 36 hours (Q) or 4 days (R) after the induction of germination. All tissues were stained for GUS activity overnight.

when compared with the *SPA1* promoter. Exceptions are the *CAB3* promoter in dark-grown seedlings and the *RolC* promoter in dark- and light-grown seedlings, which caused lower levels of GUS-SPA1 accumulation than did the *SPA1* promoter.

Expression of GUS-SPA1 in the phloem affects the development of dark- and light-grown seedlings

SPA1::GUS-SPA1 expression fully complemented the constitutive photomorphogenesis exhibited by dark-grown *spa1 spa2 spa3* mutant seedlings (Fig. 8A,B). 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. These transgenic seedlings displayed closed cotyledons but no apical hook. Epidermis-specific expression of *GUS-SPA1* in *ML1::GUS-SPA1* transgenic seedlings caused minor effects on hypocotyl elongation and partial cotyledon closure in darkness. Mesophyll-specific expression of *GUS-SPA1* (*CAB3*) also had a minor effect on hypocotyl elongation but no effect on the cotyledon phenotype. Meristem- and root-specific expression of *GUS-SPA1* (*KNAT1* and *TobRB7*, respectively) did not rescue any aspects of the *spa* triple mutant phenotype (Fig. 8A,B).

When grown in weak red light, *SPA1::GUS-SPA1* transgenic seedlings showed full complementation of the *spa1 spa2 spa3* seedling phenotype. Phloem-specific expression of *GUS-SPA1* (*SUC2* or *RolC*) partially complemented the hypocotyl-length phenotype of the *spa1 spa2 spa3* triple mutant (Fig. 8C,D), which is in contrast to the almost full complementation observed in dark-grown seedlings (Fig. 8A,B). In red light, *SUC2* conferred a higher degree of complementation than *RolC*, which is consistent with the higher level of GUS-SPA1 accumulation in *SUC2::GUS-SPA1* than in *RolC::GUS-SPA1* seedlings (Fig. 7D,R). Mesophyll- and epidermis-specific expression of *GUS-SPA1* had slight effects on the *spa1 spa2 spa3* mutant phenotype in red light. Neither shoot apical meristem- nor root-specific expression of *GUS-SPA1* rescued the parental *spa* triple mutant phenotype (Fig. 8C,D).

Taken together, these data show that expression of SPA1 in phloem tissues strongly promotes seedling etiolation, in particular in dark-grown seedlings. Because this result at first sight seemed surprising, we considered the possibility that undetectable GUS-SPA1 expression in non-phloem tissues causes the rescue of the *spa* mutant phenotype. However, as high-level GUS-SPA1 expression

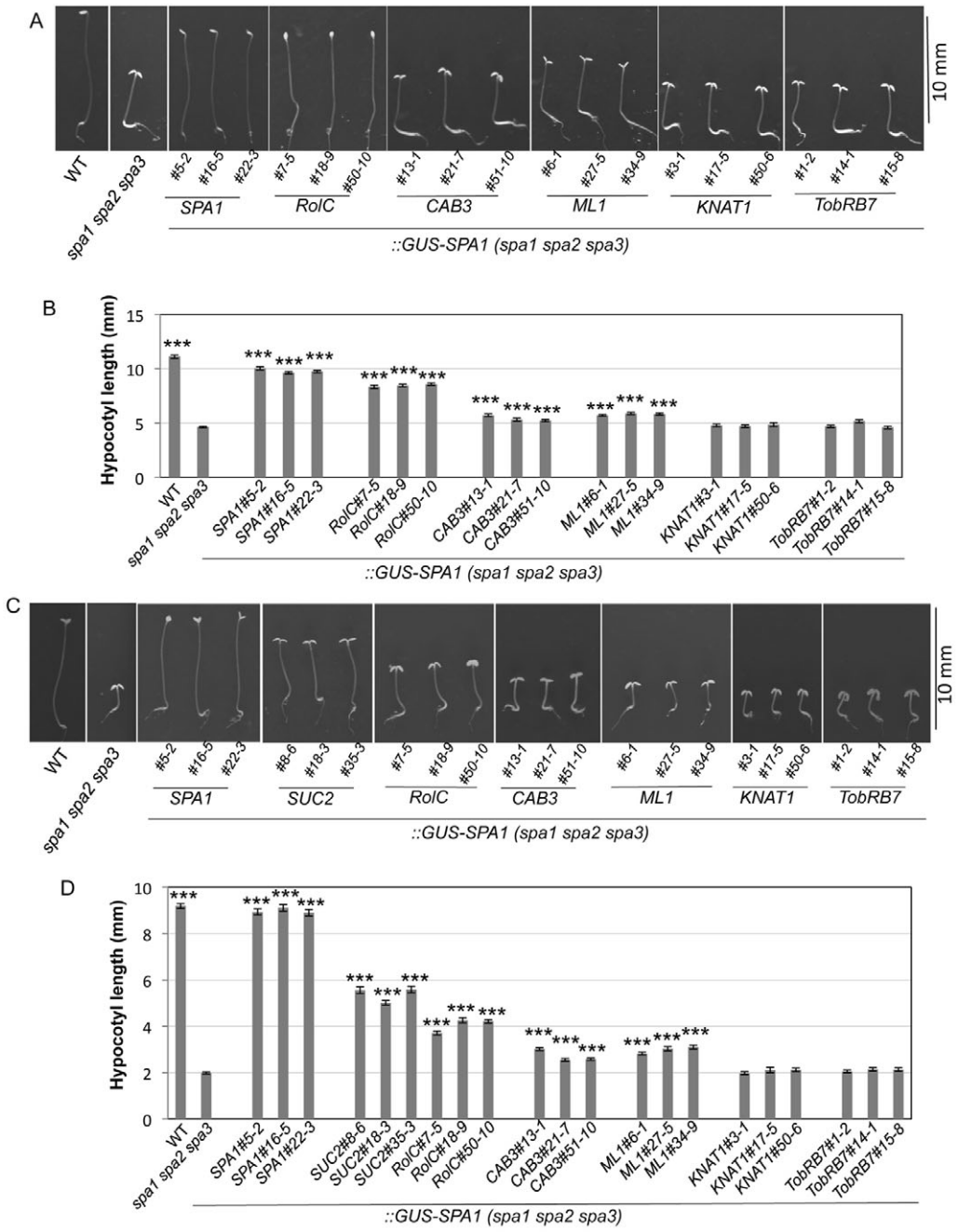

Fig. 8. SPA1 activity in the phloem alters the development of dark- and light-grown seedlings. (**A**,**C**) Visual phenotype 4-day-old seedlings grown in darkness (A) or weak Rc (0.1 μmol m^{-2} s^{-1}) (C). Shown are the wild type (WT) and the *spa1 spa2 spa3* mutant, as controls, and transgenic *spa1 spa2 spa3* mutants expressing GUS-SPA1 under the control of the indicated promoters. For each transgene, three independent transgenic lines are shown. (**B**,**D**) Quantification of the hypocotyl length of seedlings grown in darkness (B) or weak Rc (0.1 μmol m^{-2} s^{-1}) (D). Genotypes are as in A,C. Asterisks indicate significant differences between the indicated genotypes and the *spa1 spa2 spa3* mutant (*t*-test, $P<0.001$). Data are mean±s.e.m.

in none of the other tissues, especially not in the mesophyll or epidermis, affected seedling growth of the *spa* mutant in a similarly strong fashion, we regard this possibility as extremely unlikely.

SPA1 acts in the phloem to suppress stomata and pavement cell differentiation in dark-grown seedlings

Full differentiation of stomata in cotyledons requires light and is dependent on photoreceptor function (Kang et al., 2009). *cop1* and *spa1 spa2 spa3* mutants differentiate stomata also in darkness and thus display constitutive photomorphogenesis also with respect to this phenotype (Kang et al., 2009) (Fig. 9A,B). The differentiation of pavement cells in the epidermis is also suppressed in darkness through a SPA-dependent mechanism. Cotyledons of dark-grown wild-type seedlings showed small, non-lobed pavement cells, while cotyledons of the *spa1 spa2 spa3* triple mutant exhibited large, multi-lobed pavement cells in darkness similar to wild-type seedlings grown in the light (Fig. 9A,B; see Fig. S8 in the supplementary material).

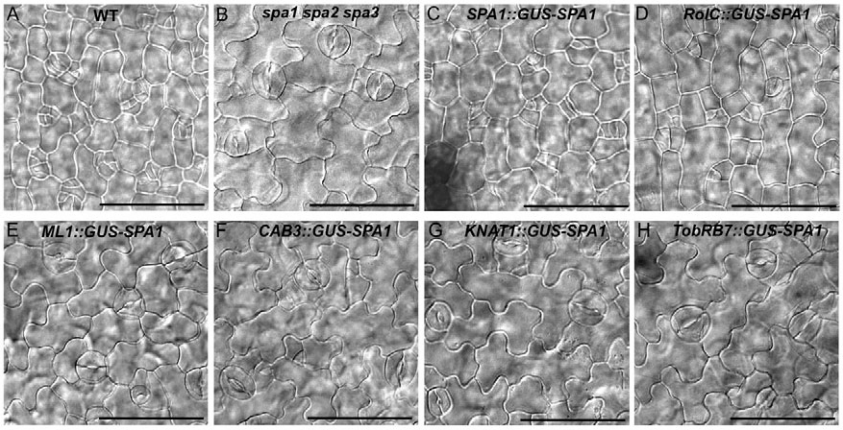

Fig. 9. SPA1 acts in the phloem to suppress the differentiation of stomata and pavement cells in dark-grown seedlings. (**A-H**) Abaxial epidermis of cotyledons of dark-grown seedlings of the wild type (A), the *spa1 spa2 spa3* mutant (B) and transgenic *spa1 spa2 spa3* mutant lines carrying the constructs *SPA1::GUS-SPA1* (line 5-2) (C), *RolC::GUS-SPA1* (line 7-5) (D), *ML1::GUS-SPA1* (line 6-1) (E), *CAB3::GUS-SPA1* (line 13-1) (F), *KNAT1::GUS-SPA1* (line 3-1) (G) and *TobRB7::GUS-SPA1* (line 1-2) (H). Scale bars: 50 μm.

SPA1::GUS-SPA1 transgenic lines showed arrested stomata development as well as non-lobed epidermal cells in cotyledons of dark-grown seedlings (Fig. 9C; see Fig. S8 in the supplementary material), indicating that SPA1 is sufficient to suppress stomata and pavement cell differentiation in the dark-grown *spa1 spa2 spa3* mutant. *RolC::GUS-SPA1* lines expressing *GUS-SPA1* exclusively in the phloem also showed arrested stomata development and non-lobed epidermal cells in darkness (Fig. 9D; see Fig. S8 in the supplementary material). Their epidermal cells appeared similar to those of dark-grown wild-type seedlings, with the exception that their pavement cells were larger than those of the wild type, but still smaller than those of the *spa1 spa2 spa3* mutant.

Expression of *GUS-SPA1* in epidermal cells (*ML1*) did not suppress stomata differentiation in the *spa* triple mutant (Fig. 9E and see Fig. S8 in the supplementary material), even though the *ML1* promoter is active in stomata (data not shown). This suggests that SPA1-dependent inhibition of stomata differentiation is not a cell-autonomous response. Shape and size of pavement cells, by contrast, was – in part – affected by SPA1 expression in the epidermis. These *ML1::GUS-SPA1* transgenic lines exhibited smaller pavement cells with reduced lobing when compared with the parental *spa* triple mutant (Fig. 9B,E; see Fig. S8 in the supplementary material). Mesophyll-, meristem- or root-specific expression of *GUS-SPA1* did not rescue the stomata or the pavement cell phenotype of the *spa* triple mutant progenitor (Fig. 9F-H; see Fig. S8 in the supplementary material).

DISCUSSION

Many light responses, such as seedling de-etiolation, shade avoidance and photoperiodic flowering, involve communication between different tissues or organs (Bou-Torrent et al., 2008; Josse et al., 2008; Montgomery, 2008). A number of recent studies have elucidated the role of light-sensing photoreceptors in various tissues of *Arabidopsis* and have therefore provided an important framework in identifying photoreceptive sites for the light-induced regulation of plant growth and development (Endo et al., 2007; Endo et al., 2005; Warnasooriya and Montgomery, 2009). In which tissues light signaling proteins function has thus far not been investigated. Here, we have examined tissue-specific functions of a key repressor of light signaling, SPA1, which suppresses photomorphogenesis in darkness by ubiquitylation and subsequent degradation of activators of the light response (Hoecker, 2005). To this end, we have expressed a GUS-SPA1 fusion protein under the control of tissue-specific promoters in transgenic plants. We show that SPA1 activity in phloem tissue of the vasculature controls seedling differentiation, leaf expansion and flowering time. Our results demonstrate that systemic signals are transmitted from the phloem to other tissues and, therefore, contribute to the coordinated growth of the different tissue layers in seedlings and leaves.

SPA1 acts in the phloem to control photoperiodic flowering

It has been known for a long time that photoperiodic stimuli are perceived by leaves and then transported via the phloem to the shoot apical meristem to induce the transition to flowering. Consistent with this observation, the photoreceptors cry2 and phyB were found to regulate flowering in the leaf and not in the shoot apical meristem. Interestingly, cry2 and phyB act in distinct tissues of the leaf: cry2 acts in the phloem, while phyB acts in the mesophyll to control flowering (Endo et al., 2007; Endo et al., 2005). Here, we demonstrate that SPA1, like cry2, operates in the phloem, but not in the mesophyll, the epidermis or the shoot apical meristem, to regulate photoperiodic flowering. Moreover, *spa1* was fully epistatic to *cry2*, indicating that SPA1 acts downstream of cry2 in the same signaling pathway. These results support the idea that light-activated cry2 inhibits the activity of the COP1/SPA complex in phloem cells (Liu et al., 2008).

In contrast to *cry2*, *phyB* showed additive interactions with *spa1* in flowering time control, suggesting that *spa1* and *phyB* act in independent pathways. This is supported by recent evidence showing that red light and phyB control the degradation of CO via a COP1-independent mechanism (Jang et al., 2008). The independent functions of SPA1 and phyB are also reflected by their sites of actions, which are different for SPA1 (phloem) and phyB (mesophyll) (Endo et al., 2005).

It was recently shown that COP1, like SPA1, can act in the phloem to control flowering time, though other tissues have not been investigated (Jang et al., 2008). Nevertheless, a function of the COP1/SPA1 complex in the phloem is reasonable because this complex controls flowering time by physically interacting with CO, which is expressed only in the vasculature and, moreover, is capable of operating only in the phloem among the tissues tested (An et al., 2004; Jang et al., 2008; Laubinger et al., 2006; Liu et al., 2008). Taken together, these results demonstrate that the COP1/SPA1 complex regulates CO stability cell-autonomously in phloem companion cells. Interestingly, the COP1/SPA complex also controls flowering time independently of CO as CO-deficient *spa1 spa3 spa4 co* and *cop1 co* mutants still exhibit a slight early-flowering phenotype (P.F. and U.H., unpublished) (Jang et al.,

2008). Our finding that SPA1 acts in the phloem and not in other tissues, therefore, indicates that the CO-independent activities of SPA1 also operate in the phloem.

SPA1 is required in both phloem and mesophyll cells to allow normal leaf expansion

spa and *cop1* mutants are very small plants that show strong defects in leaf expansion (Deng and Quail, 1992; Laubinger et al., 2004). The *spa* mutant leaf phenotype was partially rescued when GUS-SPA1 was expressed in the phloem or in the mesophyll, whereas it was fully rescued when GUS-SPA1 was expressed in both of these tissues. This demonstrates that SPA1 activity in both tissues is necessary for normal leaf expansion. Expression of GUS-SPA1 in the epidermis, by contrast, did not rescue the *spa* mutant leaf phenotype, even though the epidermis-specific promoters used were expressed at even higher levels than the native *SPA1* promoter, which expresses rather poorly in epidermal cells. This lack of SPA1 function in the epidermis is in contrast to brassinosteroid-mediated leaf expansion, which was shown to operate from the epidermis in a non-cell autonomous fashion (Savaldi-Goldstein et al., 2007). Thus, SPA1-induced signaling and brassinosteroid signaling promote leaf expansion through distinct mechanisms.

SPA1 activity in phloem as well as mesophyll cells increased division and expansion of cells in the epidermal layer. This clearly shows that there are SPA1-dependent non-cell-autonomous signal(s) derived from the phloem and the mesophyll, which trigger cell division and expansion in the epidermis. In plants, some proteins have been reported to move from cell to cell via plasmodesmata (Lucas and Lee, 2004). The SPA1 protein itself is not capable of movement because GUS activity in the GUS-SPA1 transgenic lines was not detected outside the expected expression domains. In addition, expression of GFP-SPA1 by particle bombardment never led to detectable GFP fluorescence in cells surrounding the transfected cell (data not shown). The constitutive nuclear localization of SPA1 would, moreover, most probably prevent movement of the protein (Hoecker et al., 1999). Hence, we suggest that a downstream target of the COP1/SPA1 complex initiates a systemic activity.

Such a SPA1-induced systemic signal might involve changes in hormone levels because hormones have been shown to mediate light-controlled leaf expansion. For example, the reduced leaf growth associated with the shade avoidance syndrome is caused by an auxin-mediated increase in cytokinin oxidase expression that, interestingly, is primarily observed in the vasculature of developing leaves (Carabelli et al., 2007). In addition, auxin- and brassinosteroid-responsive genes are upregulated in leaves in response to shade (Kozuka et al., 2010). This is consistent with the observed increase in auxin levels via the TAA1 pathway in response to shade (Tao et al., 2008). It will, therefore, be interesting to test whether SPA1 expression in the phloem and/or mesophyll affects hormone levels in the leaf.

The mobile signal(s) could originate from the phloem/mesophyll of the growing leaf or, in addition, from older already expanded leaves followed by transport to the young growing leaf. Signaling beyond a single organ was reported for the protein KLUH, which increases petal size beyond the individual petal (Eriksson et al., 2010). Within an organ, non-cell-autonomous control of cell division was for example mediated by cell-to-cell movement of the cyclin-dependent kinase inhibitor ICK1/KRP1 (Weinl et al., 2005).

An alternative possibility is that physical forces generated by growth in SPA1-expressing phloem and/or mesophyll tissues drive cell division and expansion in the epidermis. The existence of such a mechanical stimulus is suggested by the finding that increased expression of the cell wall relaxation protein expansin promotes leaf growth (Sloan et al., 2009). In addition, local application of expansin to the shoot apical meristem could trigger local outgrowth (Fleming et al., 1997; Pien et al., 2001).

Phloem-expressed SPA1 contributes to seedling etiolation in darkness and in the light

Our results show that expression of GUS-SPA1 in phloem tissue mostly complemented the constitutive photomorphogenesis of dark-grown *spa* mutant seedlings, while it partially rescued the *spa* mutant phenotype of light-grown seedlings. Thus, phloem tissue clearly provides a strong contribution to seedling differentiation. Phloem tissue may coordinate growth of surrounding tissue via physical forces and/or diffusible molecules, including hormones such as cytokinins, auxins or gibberellins (Nemhauser, 2008).

The increased hypocotyl length in dark-grown seedlings solely results from enhanced cell elongation and does not involve cell division (Gendreau et al., 1997). In this regard, it is noteworthy that root elongation via cell elongation requires DELLA signaling in the endodermis, an inner tissue surrounding the vascular bundles of the root. It has been shown that expression of the non-degradable DELLA protein GAI in the endodermis reduced cell elongation not only of endodermal cells but also of adjacent cortical and epidermal cells. By contrast, when GAI was targeted to other cells no effect on cell expansion was observed (Ubeda-Tomas et al., 2008). Hence, the non-cell-autonomous effect of phloem-expressed SPA1 on cell elongation in dark-grown seedlings might involve a similar mechanism, possibly physical force, as endodermis-expressed GAI.

Expression of GUS-SPA1 in mesophyll tissue of the cotyledon had only a very slight affect on the *spa* mutant phenotype in dark- and in red light-grown seedlings, even though GUS-SPA1 was expressed at high levels at least in light-grown seedlings. This finding appeared surprising because it was shown previously that the phytochromes act in the mesophyll to control seedling de-etiolation in the light (Endo et al., 2005; Warnasooriya and Montgomery, 2009). Although, in darkness, no photoreceptors are active and principally different sites of action of SPA1 and the photoreceptors are conceivable, it is thought that in light-grown seedlings photoreceptors inactivate the COP1/SPA complex, possibly via direct physical interaction, to allow de-etiolation to occur. This would imply that photoreceptors and the COP1/SPA1 complex be active in the same cell. We therefore do not exclude the possibility that phytochromes and SPA1 act in both tissues, the phloem and the mesophyll. Thus, expression of SPA1 and phytochromes under the control of tissue-specific promoters/enhancers might not fully reflect the endogenous level or timing of expression in the particular tissue and, therefore, might fail to complement the respective mutant phenotype. Alternatively, phytochrome action in the mesophyll of light-grown seedlings might cell-autonomously affect PIF proteins and not the COP1/SPA complex, at least with respect to seedling de-etiolation. Active phytochromes were shown to initiate seedling de-etiolation by directly interacting with PIF proteins which are subsequently phosphorylated and degraded in a COP1-independent fashion (Leivar and Quail, 2010). In this scenario, Phytochrome-mediated reduction in COP1/SPA activity might not solely be a cell-autonomous action but might also involve cell-cell communication between phytochrome in the mesophyll and SPA1 in the phloem. This communication might involve

hormones such as gibberellins, which have been shown to control COP1 activity towards the substrate HY5 (Alabadi et al., 2008; Alabadi et al., 2004).

Interestingly, expression of the photoreceptor cry2 in the mesophyll did not rescue the cry2 mutant seedling phenotype, suggesting that phytochromes and cryptochromes act in different tissues of the seedling or, alternatively, that cry2 expression in additional tissues is required (Endo et al., 2007). As cry2 is also thought to inactivate the COP1/SPA complex, it is likely that multiple mechanisms of light-induced seedling de-etiolation exist that might occur in different tissues of the seedling. In summary, we propose that seedling de-etiolation is a result of complex regulatory mechanisms involving mesophyll, phloem and, possibly, additional tissues.

Expression of GUS-SPA1 in the phloem was also sufficient to suppress stomata and pavement cell differentiation in epidermal cells of dark-grown seedlings. By contrast, expression of GUS-SPA1 in the epidermis did not alter stomata differentiation, but slightly affected pavement cell morphology. This indicates that SPA1 acts non-cell-autonomously in these responses. It has been shown previously that COP1 inhibits stomata differentiation upstream of the MAPKKK YDA and in parallel with the receptor-like protein TMM (Kang et al., 2009). The COP1/SPA complex might thus regulate stomata development through other receptor-like kinases, such as the ER family, or, alternatively, through hormonal (Saibo et al., 2003) or other thus far unknown mechanisms.

In summary, our results show that phloem tissue is a major site of action of SPA1. Consistent with this finding, SPA1 accumulates to particularly high levels in vascular tissue when compared with other tissues. Nevertheless, the SPA1 protein is expressed in most tissues of the seedling and the adult plant. This suggests that SPA1 has additional functions in these tissues that might be controlled in a cell-autonomous manner. Unraveling cell-autonomous and non cell-autonomous functions of SPA1, as well as of photoreceptors and other signaling intermediates will be an interesting endeavor in the future.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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