Development 133, 4608 (2006) doi:10.1242/dev.02691

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There was an error published in *Development* **133**, 3213-3222.

The name of author José Le Gourrierec was incorrectly listed as José Gentilhomme.

The authors apologise to readers for this mistake.

Development 133, 4391 (2006) doi:10.1242/dev.02661

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The corresponding author's e-mail address was published incorrectly. The correct address is: hoeckeru@uni-koeln.de.

We apologise to authors and readers for this mistake.

Development 133, 3213-3222 (2006) doi:10.1242/dev.02481

Arabidopsis SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability

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The four-member SPA protein family of *Arabidopsis* acts in concert with the E3 ubiquitin ligase COP1 to suppress photomorphogenesis in dark-grown seedlings. Here, we demonstrate that SPA proteins are, moreover, essential for photoperiodic flowering. Mutations in *SPA1* cause phyA-independent early flowering under short day (SD) but not long day (LD) conditions, and this phenotype is enhanced by additional loss of *SPA3* and *SPA4* function. These *spa1 spa3 spa4* triple mutants flower at the same time in LD and SD, indicating that the SPA gene family is essential for the inhibition of flowering under non-inductive SD. Among the four SPA genes, *SPA1* is necessary and sufficient for normal photoperiodic flowering. Early flowering of SD-grown *spa* mutant correlates with strongly increased *FT* transcript levels, whereas *CO* transcript levels are not altered. Epistasis analysis demonstrates that both early flowering and *FT* induction in *spa1* mutants is fully dependent on *CO*. Consistent with this finding, SPA proteins interact physically with CO in vitro and in vivo, suggesting that SPA proteins regulate CO protein function. Domain mapping shows that the SPA1-CO interaction requires the CCT-domain of CO, but is independent of the B-box type Zn fingers of CO. We further show that *spa1 spa3 spa4* mutants exhibit strongly increased CO protein levels, which are not caused by a change in *CO* gene expression. Taken together, our results suggest, that SPA proteins regulate photoperiodic flowering by controlling the stability of the floral inducer CO.

KEY WORDS: SPA1, Photomorphogenesis, Flowering time, Photoperiodism, CONSTANS, *Arabidopsis*

INTRODUCTION

Plants use light as a source of information to optimally adapt growth and development to the ambient environment. Light is perceived by the plant through several classes of photoreceptors: the red light (R)- and far-red light (FR)-absorbing phytochromes; the blue light (B)/UV-A responsive cryptochromes and phototropins as well as thus far uncharacterized UV-B receptors (Briggs and Spudich, 2005; Chen et al., 2004). The recently identified ZEITLUPE protein family may also contribute to blue light perception (Imaizumi et al., 2003). A dramatic effect of light is observed during seedling development. Suppression of the light response in dark-grown *Arabidopsis* seedlings requires the activities of CONSTITUTIVELY PHOTOMORPHOGENIC (COP1) and members of the SUPPRESSOR OF *PHYA-105* (SPA) protein family. *spa* and *cop1* mutants, therefore, undergo constitutive photomorphogenesis and display features of light-grown seedlings even when grown in complete darkness (Laubinger et al., 2004; Osterlund et al., 1999). COP1, a RING-finger containing WD-repeat protein, functions as an E3 ubiquitin ligase. It suppresses photomorphogenesis in darkness by ubiquitinating activators of the light response, such as the transcription factors LONG HYPOCOTYL 5 (HY5), LONG AFTER FR 1 (LAF1) and LONG HYPOCOTYL IN FR 1 (HFR1), which are subsequently degraded by the proteasome (Duek et al., 2004; Jang et al., 2005; Osterlund et al., 2000; Seo et al., 2003; Yang et al., 2005b). In the light, activated photoreceptors are thought to inhibit COP1 function so that these transcription factors are no longer degraded.

Members of the four-member SPA protein family contain a COP1-like WD-repeat domain, a coiled-coil domain and a kinase-like domain (Hoecker et al., 1999; Laubinger and Hoecker, 2003). They function redundantly in suppression of photomorphogenesis in darkness. Thus, strong constitutive photomorphogenesis is observed only when all four SPA genes are defective (Laubinger et al., 2004). *spa1*, *spa3* and *spa4* single mutant seedlings, by contrast, show normal development in darkness, while photomorphogenesis in the light is enhanced in a fashion that was fully dependent on a functional phytochrome A (*PHYA*) gene (Hoecker et al., 1998; Laubinger and Hoecker, 2003; Fittinghoff et al., 2006). SPA proteins are also important for normal adult growth because *spa* quadruple mutants exhibit extreme dwarfism (Laubinger et al., 2004). Further genetic analyses indicated that the individual SPA genes have overlapping but distinct functions during plant development. Whereas *SPA3* and *SPA4* predominate in the regulation of adult growth, *SPA1* and *SPA2* are the primary players in suppression of photomorphogenesis in dark-grown seedlings (Laubinger et al., 2004). Differences in SPA gene expression patterns appear to contribute to the divergence in *SPA1-SPA4* function (Fittinghoff et al., 2006).

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Accepted 7 June 2006

All SPA proteins interact with COP1 in vitro, and an in vivo interaction was observed between SPA1 and COP1. Because *spa* and *cop1* mutations also interact genetically it is hypothesized that SPA proteins function in concert with COP1 to ubiquitinate activators of the light response (Hoecker and Quail, 2001; Laubinger et al., 2004; Saijo et al., 2003). In agreement with this idea, HY5 and HFR1 protein levels are increased in *spa1* mutant seedlings (Saijo et al., 2003; Yang et al., 2005a). Moreover, recombinant SPA1 altered the in vitro ubiquitin ligase activity of COP1 (Saijo et al., 2003; Seo et al., 2003).

Light also controls the transition from vegetative to reproductive development. Many plant species use day length (photoperiod) to adjust flowering time to the changing seasons (Putterill et al., 2004). As a facultative long-day plant, *Arabidopsis* flowers much earlier in long days (LD) than in short days (SD). A key component in LD-triggered flowering is the putative transcription factor CONSTANS (CO), which contains a B-Box-type Zn-finger and a conserved CCT domain (Koornneef et al., 1991; Putterill et al., 1995). CO promotes flowering by upregulating the expression of the genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF CONSTANS 1* (*SOC1*), which then in turn induce floral transition (Abe et al., 2005; Hayama and Coupland, 2004; Samach et al., 2000; Searle and Coupland, 2004; Searle et al., 2006; Wigge et al., 2005; Yoo et al., 2005). *FT* and *SOC1* are also responsive to other cues, such as extended cold treatment in vernalization-requiring accessions, as well as developmental age (autonomous pathway), indicating that *FT* and *SOC1* integrate several flowering-time pathways (Putterill et al., 2004). It appears, however, that *FT* expression is primarily regulated by photoperiod, whereas the expression of *SOC1* is more strongly regulated by the vernalization/autonomous pathway than by the photoperiod pathway (Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000; Samach et al., 2000; Wigge et al., 2005).

The expression of *CO* is regulated by the circadian clock, with *CO* transcript levels rising around 12 hours after dawn. Therefore, high levels of *CO* transcript occur at the end of daytime in LD but during night time in SD (Suarez-Lopez et al., 2001). The 'coincidence model' thus proposes that LD can trigger flowering because the expression of *CO* coincides with the exposure of plants to light (Roden et al., 2002; Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). Recent results provided molecular support for this hypothesis by showing that CO is also regulated at the post-transcriptional level: CO protein is stabilized by light, while in darkness it is rapidly degraded in the proteasome (Valverde et al., 2004). The combination of circadian regulation of *CO* transcript levels and light-induced stabilization of the CO protein ensures that the CO protein accumulates exclusively under inductive LD conditions. CO protein accumulation in the light is dependent on the light quality because CO accumulates in FR and B, but not in R (Valverde et al., 2004). This correlates well with the knowledge that the FR-perceiving photoreceptor phyA and the B-responsive cryptochrome 2 (cry2) promote flowering in LD, while the R-photoreceptor phytochrome B (phyB) inhibits flowering (Hayama and Coupland, 2004; Searle and Coupland, 2004).

Besides the photoreceptors, light signaling intermediates also affect photoperiodic flowering. *cop1* mutants show no delay in flowering under SD conditions, indicating that COP1 is required for the suppression of flowering in non-inductive SD (McNellis et al., 1994). When grown in the presence of sucrose, *cop1* mutants flowered even in complete darkness, while wild-type plants never bolted under these conditions (Nakagawa and Komeda, 2004). The molecular nature of COP1 function in the control of flowering time, however, is unknown. Because SPA proteins function in concert with COP1, and, moreover, suppress seedling light responses in darkness, we were interested in examining the roles of SPA genes in the light regulation of flowering time using genetic and molecular approaches.

MATERIALS AND METHODS

Plant material

The mutants *spa1-2, spa1-3* and *spa1-2 phyA-101* were generated in the RLD accession and are described by Hoecker et al. (Hoecker et al., 1998). The mutant *phyA-101* (RLD) is described elsewhere (Dehesh et al., 1993). All spa mutants in the Col accession have been described previously (Fittinghoff et al., 2006; Laubinger and Hoecker, 2003; Laubinger et al., 2004). The *co-SAIL* allele (Col) was obtained from the SAIL T-DNA collection (Sessions et al., 2002) and was confirmed to carry a T-DNA insertion 342 bp after the ATG. It causes late flowering in LD very similar to other *co* mutations (Koornneef et al., 1991; Putterill et al., 1995).

To generate the *spa1-7 co-SAIL* double mutant, a segregating F2 population was grown in FR and short, i.e. *spa1-7* mutant, seedlings were selected and transferred to LD conditions. Plants that flowered late were confirmed to be homozygous *spa1-7 co* mutant using PCR-based markers that can distinguish between the respective mutant and wild-type alleles.

Analysis of flowering time

To determine the flowering time, seeds were sown directly onto soil and plants were grown in a randomized fashion in either SD (8 hours light/16 hours darkness) or LD (16 hours light/8 hours darkness) at 21°C. The light source were fluorescent tubes (80 μmol m^{-2} s^{-1}). Experiments that included the *phyA-101* and the *spa1-2 phyA-101* mutants were conducted in either SD (8 hours fluorescent light/16 hours darkness) or SD+extension (8 hours fluorescent light/8 hours incandescent light/8 hours darkness) at 21°C. Light was provided by fluorescent white-light tubes (200 μmol m^{-2} s^{-1}) and incandescent 60W bulbs. Flowering time was scored by determining the number of rosette leaves when the first inflorescence was seen by eye.

Analysis of transcript levels

Total RNA was isolated from the green parts of soil-grown plants 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). cDNAs were diluted to 100 μl with water and 2 μl of diluted cDNA was used for PCR-amplification of *CO*, *FT*, *SOC1*, *FLC*, *SPA1*, *SPA2*, *SPA3*, *SPA4* and *UBQ10* fragments using gene-specific primers. The *UBQ10* fragment was used as a control to normalize for the amount of cDNA used. *CO*, *FT*, *UBQ10* and *SOC1* and *FLC* primers were described previously (Blazquez and Weigel, 1999; Mockler et al., 2004). A *SPA1* fragment was amplified using 5′TGGTCATGAGAAAGCGGTGA3′ and 5′CCTCCAACAGACGCTCGAC3′; *SPA2* was amplified using 5′GCAGTTAGCTATGCGAAGTTC3′ and 5′GCAAACGCTTGAAACGAACAGG3′; *SPA3* was amplified using 5′GAGAAAGGAGTCTACAATAAGTTG3′ and 5′CTCATTGATGGTCGACAAGTTGGCTCA3′; and *SPA4* was amplified using 5′TGAAGAAGATAATGGTTCTCTGTG3′ and 5′CTCATCGATGGTCGACAGCTA3′. For all cDNAs, the exponential range of amplification was determined experimentally. Then, 25 (for *CO*), 28 (*FT*), 20 (*SOC1*), 20 (*FLC*), 20 (*SPA1*), 20 (*SPA2*), 22 (*SPA3*), 24 (*SPA4*) and 17 (*UBQ10*) cycles were used in all experiments. PCR reactions with each cDNA and primer pair were carried out three times simultaneously. The three PCR products were pooled, separated on an agarose gel, transferred to a Nylon membrane and hybridized with a radioactively labeled gene-specific probe. Hybridization signals were quantified by phosphorimager analysis.

In vitro binding assays

Constructs expressing GAD-SPA1, GAD-SPA2, GAD-SPA3 or GAD-SPA4 have been described previously (Hoecker and Quail, 2001; Laubinger et al., 2004; Laubinger and Hoecker, 2003). All constructs for expression of CO (without His-tag) were generated by PCR-amplifying the full open-reading-frame (ORF) of *CO* or parts of the ORF of *CO* using primers with restriction sites and subsequent ligation of the digested PCR products into the *Nco*I-

*Bam*HI restriction sites of the vector pET15b (Novagen). CO expresses the full-length CO protein (373 amino acids), COΔVP3 the amino acids 1-364, CO-CCT the amino acids 272-373 and COΔB-Box the amino acids 107-373. To express His_6-CO protein, the ORF of *CO* was inserted into the vector pDEST17 (Invitrogen) by Gateway recombination cloning. His_6-COΔCCT expresses amino acids 1-331 of CO, COmVP1-3 expresses a full-length CO protein in which three VP motifs (amino acids 214-215, 265-266 and 370-371) were changed to two alanine residues by site-directed mutagenesis (Quickchange kit, Stratagene, La Jolla, USA).

All proteins were synthesized using the TnT reticulocyte coupled transcription and translation system (Promega) in the presence of ^{35}S-labelled methionine. Prey proteins were fully labeled with ^{35}S-methionine, while bait proteins were synthesized in the presence of a mixture of labeled and unlabelled methionine in order to facilitate protein detection after SDS-PAGE. Protein synthesis and co-immunoprecipitations were conducted as described previously (Hoecker and Quail, 2001). Briefly, 10 μl each of bait and prey TnT reactions were added to 200 μl of binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiotreitol (DTT), 0.1% Tween 20) and incubated on a rotary platform for 1-2 hours at 4°C. Co-immunoprecipitation were conducted by subsequently adding 0.4 μg of α-GAD antibody (Santa Cruz Biotechnology) and 8 μl of protein A-coated magnetic beads (Dynal, Oslo, Norway). Precipitates were washed four times with 1 ml of binding buffer (without DTT). Pellet and supernatant fractions were resolved by SDS-PAGE and visualized using a phosphorimager (Fuji).

Confocal microscopy, SPA1-CO colocalization and FRET analysis

To express CFP-SPA1 and YFP-CO in plants, the open-reading-frames of SPA1 or CO, respectively, were amplified by PCR and cloned into the GATEWAY vectors pENSG-CFP or pENSG-YFP by recombination cloning. In these vectors, CFP-SPA1 and YFP-CO are expressed from the constitutive 35S-promoter.

Laser-scanning confocal microscopy was performed using a Leica TCS SP2 system (Leica Microsystems, Heidelberg, Germany). YFP was excited with the 514 nm line and CFP was excited with the 405 nm line of a diode laser of an argon laser 20%. Images were taken with an objective HC PL APO CS 20.0×0.70 UV. Fluorescence was detected in case of YFP between 525-590 nm and in case of CFP between 454-503 nm.

For SPA1-CO co-localization studies and FRET acceptor photobleaching, *Arabidopsis* leaf epidermal cells of 3-week-old, LD-grown plants were co-transfected ballistically with two plasmid constructs, respectively, encoding CFP-SPA1, YFP-CO, CFP or YFP, and analyzed 24 hours after bombardment. Cells exhibiting co-expression of both fluorescent proteins were bleached in the acceptor YFP channel by scanning an ROI (region of interest) with 100% laser intensity. FRET efficiency was calculated directly by the TCS software using the following formula: $FRET_{Eff}=(D_{post}-D_{pre})/D_{post}$ for all $D_{post}>D_{pre}$.

CO protein detection

Nuclear extracts were prepared from 12-day-old, LD-grown (16 hour light/8 hour dark) plants at zeitgeber (ZT) 16 as described previously (Valverde et al., 2004). Nuclear proteins were separated by SDS-PAGE using 10% bis-Tris NuPAGE ready-cast gels (Invitrogen), transferred to a nitrocellulose membrane and probed with an anti-CO antibody followed by an horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized by ECL (Pierce). The membrane was subsequently reprobed with an antibody against histone H3a (Abcam) to control for unequal loading.

RESULTS

spa1 mutants flower early in SD but not in LD

To investigate whether SPA genes play a role in the control of flowering time, we grew wild-type plants and mutant plants defective in individual SPA genes under SD and LD conditions. Fig. 1A and Table 1 show that SD-grown *spa1* mutants in both accessions, RLD and Col, flowered much earlier than the respective wild-type plants. Under LD conditions, by contrast, *spa1* mutant plants flowered at the same time as wild-type plants. Thus, *spa1* mutants exhibited a defect specifically under SD conditions. Early flowering of *spa1* mutants was more pronounced in the RLD accession than in Col, indicating that the genetic background influences SPA1 function.

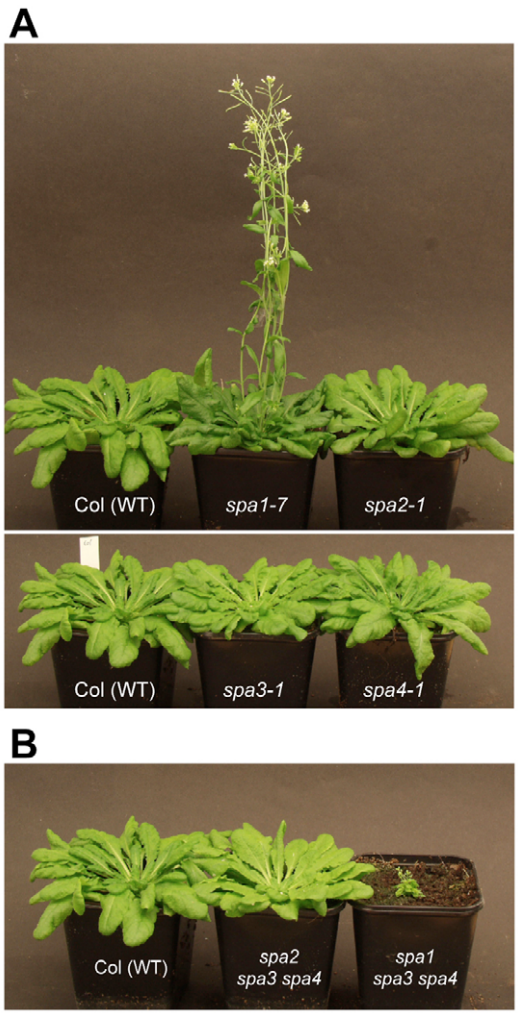

Fig. 1. Visual phenotype of 78-day-old wild-type, *spa* single and *spa* triple mutants grown in SD. (**A**) *spa1* mutants, but not *spa2*, *spa3* and *spa4* single mutants, flower early in SD. (**B**) A functional *SPA1* gene, but not *SPA2*, is sufficient for normal flowering in SD.

Mutations in other SPA genes did not cause a change in flowering time, either in SD or in LD (Fig. 1A, Table 1). This suggests that, among the SPA genes, *SPA1* has a predominant role in controlling flowering time.

Early flowering in SD-grown *spa1* mutants is independent of phyA

It has been shown previously that mutations in individual SPA genes (*spa1*, *spa3* or *spa4*) cause enhanced seedling responsiveness to light only when a wild-type *PHYA* gene is present (Fittinghoff et al., 2006; Hoecker et al., 1998; Laubinger and Hoecker, 2003). We therefore investigated whether early flowering of SD-grown *spa1* mutants is also phyA dependent. Table 2 shows that *spa1 phyA* mutant plants flowered much earlier than *phyA* mutant plants, indicating that a *spa1* mutation causes early flowering in SD even in the absence of a functional *PHYA* gene. This is consistent with the finding that *phyA* mutations do not alter flowering time under SD conditions (Reed et al., 1994).

Table 1. Flowering time of *spa* single and triple mutants in SD and LD

Genotype	Leaf number (SD)	Leaf number (LD)
RLD (wild type)	73.2±1.9	10.9±0.3
spa1-2	13.6±0.4	10.7±0.4
spa1-3	13.5±0.4	10.9±0.4
Col (wild type)	105.7±2.8	19.1±0.5
spa1-7	46.6±1.0	20.3±0.7
spa2-1	101.2±3.3	19.0±0.8
spa3-1	107.5±4.8	18.8±0.9
spa4-1	99.2±3.0	19.8±1.1
spa1 spa3 spa4	14.8±0.4	16.0±0.8
spa2 spa3 spa4	119.6±4.6	18.4±0.4

Plants were grown in short day (SD, 8 hour light/16 hour dark) or long days (LD, 16 hour light/8 hour dark). Values shown are mean numbers of rosette leaves at flowering ±1 s.e.m. At least 15 plants were analyzed for each genotype. The mutants *spa1-2* and *spa1-3* are in the RLD accession, all other mutants are in the Col accession.

Table 2. Early flowering of *spa1* mutants in SD does not require phyA

Genotype	Leaf number (SD)	Leaf number (SD+extension)
RLD (wild type)	35.9±1.7	10.2±0.3
spa1-2	13.2±0.6	7.3±0.2
phyA-101	34.3±2.6	22.1±1.9
spa1-2 phyA-101	17.3±1.3	10.6±0.4

Plants were grown in short day (SD, 8 hour light/16 hour dark) or short day + extension (8 hour fluorescent light/8 hour incandescent light/8 hour darkness). Values shown are mean numbers of rosette leaves at flowering ±1 s.e.m. At least 15 plants were analyzed for each genotype.

To further assess a possible function of phyA, we grew plants under daily cycles of 8 hours fluorescent light + 8 hours incandescent light + 8 hours darkness. The FR-rich incandescent light causes phyA-dependent photoperiodic flowering (Johnson et al., 1994). Also under this light regime, *spa1 phyA* mutant plants flowered earlier than the *phyA* mutant.

SPA1 is sufficient for normal photoperiodic flowering

Though *spa2*, *spa3* and *spa4* single mutants show normal flowering (Table 1, Fig. 1A), a contribution of these SPA genes to the control of flowering time may be masked by functional redundancy. We therefore tested *spa* triple mutants for their flowering time in response to SD. *spa1 spa3 spa4* triple mutants that lacked *SPA3* and *SPA4* function in addition to *SPA1* function flowered even earlier than did the *spa1-7* single mutant (Table 1, Fig. 1B). Thus, in the absence of *SPA1* function, the loss of *SPA3* and *SPA4* function further de-repressed flowering in SD. This triple mutant flowered at a similar time in LD and SD, indicating a complete loss of photoperiodic control of flowering time. Though early flowering of the *spa1 spa3 spa4* triple mutant was most pronounced in SD, this mutant also flowered slightly earlier than the wild type in LD (Table 1).

Taken together, these results show that *SPA1* predominates in the control of flowering, *SPA3* and *SPA4* contribute somewhat, and *SPA2* plays only a minor role – if any – in this response. Indeed, *SPA1* was not only necessary but also sufficient for normal flowering in SD as the *spa2 spa3 spa4* triple mutant retained normal photoperiodic control of flowering under the conditions used (Table 1, Fig. 1B).

spa mutations cause an increase in *FT* transcript levels without a change in *CO* mRNA levels

Photoperiodic flowering requires the coincidence between expression of the flowering time gene *CO* and exposure of plants to light ('coincidence model') (Roden et al., 2002; Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). To test whether *spa* mutants flower early in SD because of a change in the *CO* expression pattern, we examined *CO* transcript levels in wild-type and *spa* mutants over a 24 hour period. *spa* mutations did not cause any dramatic changes in *CO* transcript levels (Fig. 2A,B). Wild-type, *spa1* mutant, *spa1 spa3 spa4* and *spa2 spa3 spa4* mutants showed very similar diurnal regulation of *CO* mRNA levels in SD-grown plants. This result is thus not consistent with the hypothesis that *spa* mutants flower early in SD as a result of a change in the level or pattern of *CO* expression.

We subsequently investigated whether signaling downstream of *CO* is altered in *spa* mutants. CO induces the expression of the flowering time genes *FT* and *SOC1* (Samach et al., 2000; Searle et al., 2006; Yoo et al., 2005). A comparative analysis of *FT* transcript levels in SD-grown wild-type and *spa1-7* mutant plants shows that the *spa1-7* mutation caused an ~70-fold increase in *FT* mRNA abundance relative to the wild type (ZT 16 and ZT 20) (Fig. 2A,C). A similarly strong increase in *FT* transcript levels was observed in *spa1-3* mutant plants when compared with RLD wild-type plants (data not shown). In the very early flowering *spa1 spa3 spa4* triple mutant, *FT* mRNA levels were even more drastically elevated (~1000-fold higher than in the wild type). The normal-flowering *spa2 spa3 spa4* triple mutant which has a functional *SPA1* gene, by contrast, did not show increased *FT* transcript levels (Fig. 2A,C). Thus, the flowering time of SD-grown *spa1* and *spa1 spa3 spa4* mutants correlated well with the amount of *FT* mRNA produced.

FT transcript levels in *spa* mutants were strongly elevated only during the night phase and not during day time (Fig. 2A,C). Hence, it is evident that *FT* expression in *spa* mutants followed the expression of *CO* and thus might be dependent on CO. Moreover, the pattern of *FT* mRNA abundance observed in SD-grown, early flowering *spa* mutants was very similar to that described for LD-grown wild-type plants (Suarez-Lopez et al., 2001).

CO also activates the expression of *SOC1*, a transcription factor containing a MADS-box domain (Hepworth et al., 2002; Samach et al., 2000; Searle et al., 2006; Yoo et al., 2005). However, we found that *SOC1* transcript levels were not altered in *spa1-7* mutants (Fig. 2A,D) or in *spa1-3* mutants (data not shown) when compared with the respective wild type. Thus, *spa1* mutants did not flower early in SD as a result of a change in *SOC1* transcript abundance. In both triple mutants examined, the early-flowering *spa1 spa3 spa4* mutant and the normal-flowering *spa2 spa3 spa4* mutant, *SOC1* levels were somewhat higher than in the wild type (at the most five-fold). Because this slight rise did not correlate with the flowering time of these mutants, it is unlikely that the *spa1 spa3 spa4* mutant flowered early due to increased *SOC1* expression.

Flowering time is also regulated by the repressor FLOWERING LOCUS C (FLC), which suppresses the expression of *FT* and *SOC1* in a pathway unrelated to photoperiodic flowering (Hepworth et al., 2002; Lee et al., 2000). We investigated whether the elevated *FT* transcript levels observed in the *spa1* and *spa1 spa3 spa4* mutants might be caused by a reduction in *FLC* levels. Fig. 2A,E shows that the *spa1-7* mutation did not alter *FLC* transcript abundance, although both examined triple mutants exhibited even a slight increase in *FLC* mRNA levels. This increase, like the slight elevation in *SOC1* transcript levels, did not correlate with the flowering time of SD-grown *spa* mutants, and, moreover, according to the present knowledge on

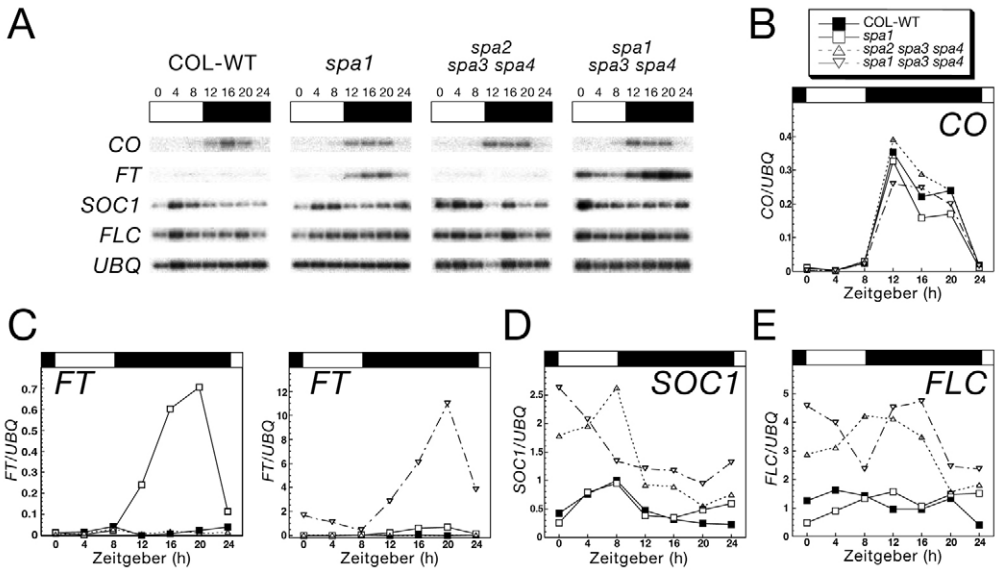

Fig. 2. *FT* transcript levels are drastically increased in SD-grown, early flowering *spa* mutants. (**A**) RT-PCR analysis of *CO*, *FT*, *SOC1*, *FLC* and *ubiquitin* (*UBQ10*) mRNA abundance in wild-type (WT) and *spa* mutant plants. (**B-E**) Quantification of mRNA abundance of the blots shown in A. The transcript levels of flowering time genes were normalized by the transcript levels of *UBQ10*. A representative experiment of two or three independent experiments is shown. Notice the difference in scale of *FT/UBQ10* in the two graphs in C. Wild-type (Col), *spa1-7*, *spa1-7 spa3 spa4* and *spa2 spa3 spa4* (Col) plants were grown in SD for 25 days. RNA was isolated from these plants and used for RT-PCR analysis.

FLC function, should lead to an inhibition of flowering. Hence, taken together, the transcript analyses demonstrate that solely the dramatic increase in *FT* transcript levels in *spa* mutants relative to the wild type correlated well with the early flowering phenotype of *spa* mutants. This suggests that *spa* mutations de-repress flowering in SD due to an inappropriate induction of *FT* expression.

Analysis of *SPA1*, *SPA2*, *SPA3* and *SPA4* transcript levels in SD-grown plants

To investigate the expression behavior of SPA genes under SD conditions, we determined the levels of all four SPA transcripts throughout a 24-hour period. Fig. 3A,B shows that *SPA1* transcript abundance shows a clear increase at ZT20, i.e. during the night phase. This result is in agreement with the findings described previously for *Arabidopsis* plants grown in 12-hour light/12-hour dark cycles (Harmer et al., 2000). The transcript levels of *SPA3* were also higher during the dark phase than during the light phase (Fig. 3A,D). *SPA2* and *SPA4* mRNA levels displayed only a slight diurnal regulation, with levels increasing only somewhat during the dark phase (Fig. 3A,C,E).

Early flowering of *spa1* mutants in SD is dependent on *CO*

Our finding that *spa1* mutations affect photoperiodic flowering by inducing *FT* expression without altering the transcript levels of the *FT*-regulator CO suggests that SPA1 may regulate *CO* post-transcriptionally or, alternatively, may repress *FT* independently of *CO*. To distinguish between these two possibilities, we examined the epistatic relationship between *spa1* and *co* in SD-grown plants. The early-flowering phenotype of *spa1* mutants was completely suppressed by the *co* mutation (Fig. 4A,B). In agreement with this finding, *FT* transcript levels were not increased in *spa1 co* double mutants (Fig. 4C,D). This demonstrates that precocious flowering of SD-grown *spa1* mutants is fully dependent on *CO*. In addition, the inappropriate induction of *FT* observed in *spa1* mutants requires *CO*. We therefore conclude that SPA1 function is *CO* dependent.

SPA proteins physically interact with CO in vitro and in vivo

We subsequently considered the CO protein a potential target of SPA function. To test this possibility, we investigated whether SPA proteins can physically interact with CO. Indeed, all four SPA

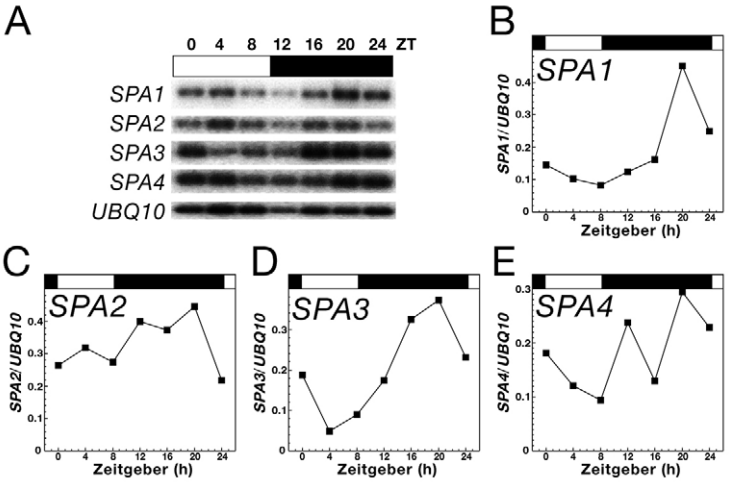

Fig. 3. Transcript analysis of *SPA1*, *SPA2*, *SPA3* and *SPA4* in SD-grown plants. (**A**) RT-PCR analysis of all four SPA genes in 25-day-old SD-grown wild-type (Col) plants. (**B-E**) Quantification of the SPA transcript abundance of the blots shown in A. The transcript levels of SPA genes were normalized by the transcript levels of *UBQ10*. A representative experiment of two independent experiments is shown.

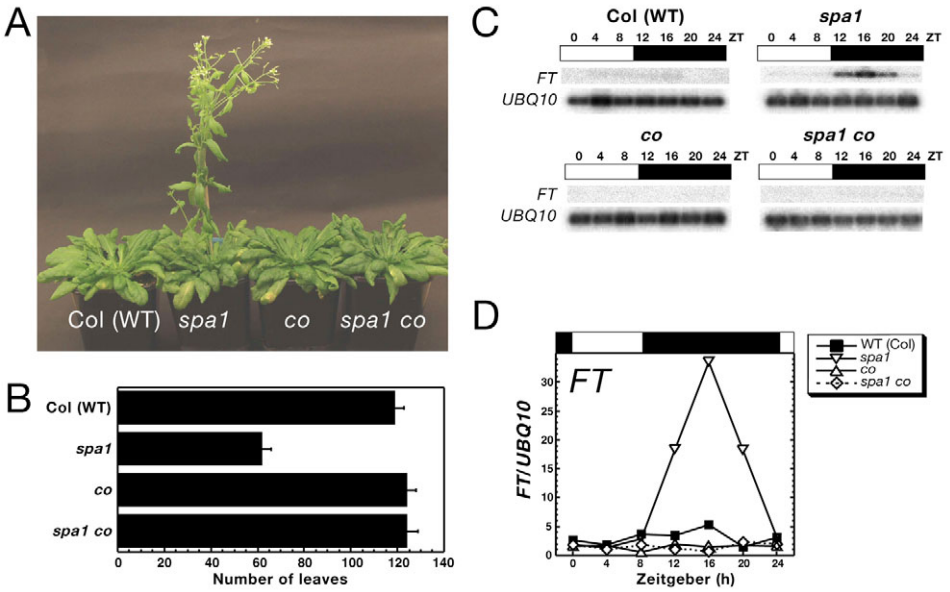

Fig. 4. Early flowering and *FT* expression in SD-grown *spa1* mutants depends on *CO*. (**A**) Visual phenotype of 83-day-old wild-type (WT), *spa1-7*, *co*, and *spa1-7 co* mutant plants grown in SD. (**B**) Flowering time in SD of genotypes shown in A. (**C**) RT-PCR analysis of *FT* and *UBQ10* transcript levels in 25-day-old plants grown in SD. (**D**) Quantification of the blots shown in C. The transcript levels of *FT* were normalized by the transcript levels of *UBQ10*.

proteins interacted with CO in an in vitro binding assay that was based on co-immunoprecipitation (Fig. 5A). We further asked whether SPA1, which is the primary regulator of photoperiodic flowering among SPA proteins, and CO also interact in vivo. To this end, we transiently co-expressed cyan fluorescent protein (CFP)-SPA1 and yellow fluorescent protein (YFP)-CO fusion proteins in leaf epidermal cells of *Arabidopsis*. Fig. 5B shows that CFP-SPA1 and YFP-CO co-localized to nuclear speckles. FRET analysis by photoacceptor bleaching further confirms that SPA1 and CO interact in planta. Significantly higher FRET efficiency was observed between CFP-SPA1 and YFP-CO when compared with controls (Fig. 5C,D).

The CCT domain of CO is essential for the interaction between SPA1 and CO

To define the SPA1-interacting domain in CO, we tested truncated versions of CO for in-vitro binding to SPA1 (Fig. 6A-C). In these experiments, some deletion-derivatives of CO were generated using a CO-protein tagged with six histidine residues. This His-tag did not affect the SPA1-binding activity of CO and is, therefore, insignificant. Deletion of both B-box type Zn fingers of CO (COΔB-Box) reduced the SPA1-binding activity of CO, but did not abolish it. This shows that the Zn fingers of CO are not essential for the interaction of CO with SPA1. C-terminal truncation of CO including part of the CCT domain (COΔCCT) abolished SPA1 binding (Fig. 6A-C), indicating that the CCT domain of CO is required for the interaction with SPA1. To determine whether the CCT domain is also sufficient for SPA1 binding, we tested a CO deletion-derivative containing only the CCT domain and the last nine amino acids of CO (CO-CCT). However, this protein was not capable of interacting with SPA1, suggesting that additional domains of CO are necessary for SPA1 binding.

CO contains 'VP motifs', which show some similarity to a COP1-binding motif detected in the transcription factors STH, STO and HY5. In HY5, this motif was also crucial for HY5 protein degradation (Holm et al., 2001). Because SPA1 and COP1 are related proteins, we considered these VP motifs of CO potential binding sites for SPA1. A deletion-derivative of CO that carries missense mutations (VP to AA) in three VP-motifs of CO (COmVP1-3) bound SPA1 as efficiently as the wild-type CO protein (Fig. 6A-C). In addition, a truncated CO protein lacking the last nine amino acids, including one VP-motif (COΔVP3), retained significant SPA1-binding activity. Thus, these VP motifs of CO were not essential for in vitro binding of CO to SPA1. However, we cannot exclude the possibility that our in vitro assay does not fully reflect the in vivo interaction between SPA1 and CO. In addition, these VP motifs may be of functional importance without affecting the SPA1-CO interaction per se.

CO protein levels are increased in *spa1 spa3 spa4* mutants

Our finding that SPA proteins interact with CO suggests that SPA proteins control CO protein function. Because SPA proteins control seedling photomorphogenesis by co-acting with the E3 ubiquitin ligase COP1, we considered the possibility that SPA proteins control CO protein degradation. We therefore examined CO protein levels in wild-type and *spa* mutant plants. CO is a protein of very low abundance and usually undetectable in nuclear extracts of wild-type plants (Valverde et al., 2004) (Fig. 7). In LD-grown *spa1 spa3 spa4* triple mutants, by contrast, CO protein accumulated to detectable levels. *CO* transcript levels were similar in wild-type and *spa* triple mutant plants, indicating that the observed increase in CO protein levels in *spa* mutants was not caused by changes in *CO* gene expression or *CO* transcript stability. Hence, the elevated CO protein abundance in *spa* mutants is most probably caused by a reduction in CO protein degradation. Consistent with this conclusion, the CO protein was previously shown to be subject to degradation via the 26S proteasome (Valverde et al., 2004).

In agreement with the elevated CO protein levels in *spa* triple mutants, these mutants flowered earlier than wild-type plants under these LD conditions (Table 1). However, although CO protein levels in *spa* triple mutants were at least as high as in a 35S-CO overexpressing line, these *spa* triple mutants flowered later than 35S-CO plants (data not shown). This implies that SPA proteins might also, directly or indirectly, be involved in regulating the activity of the CO protein.

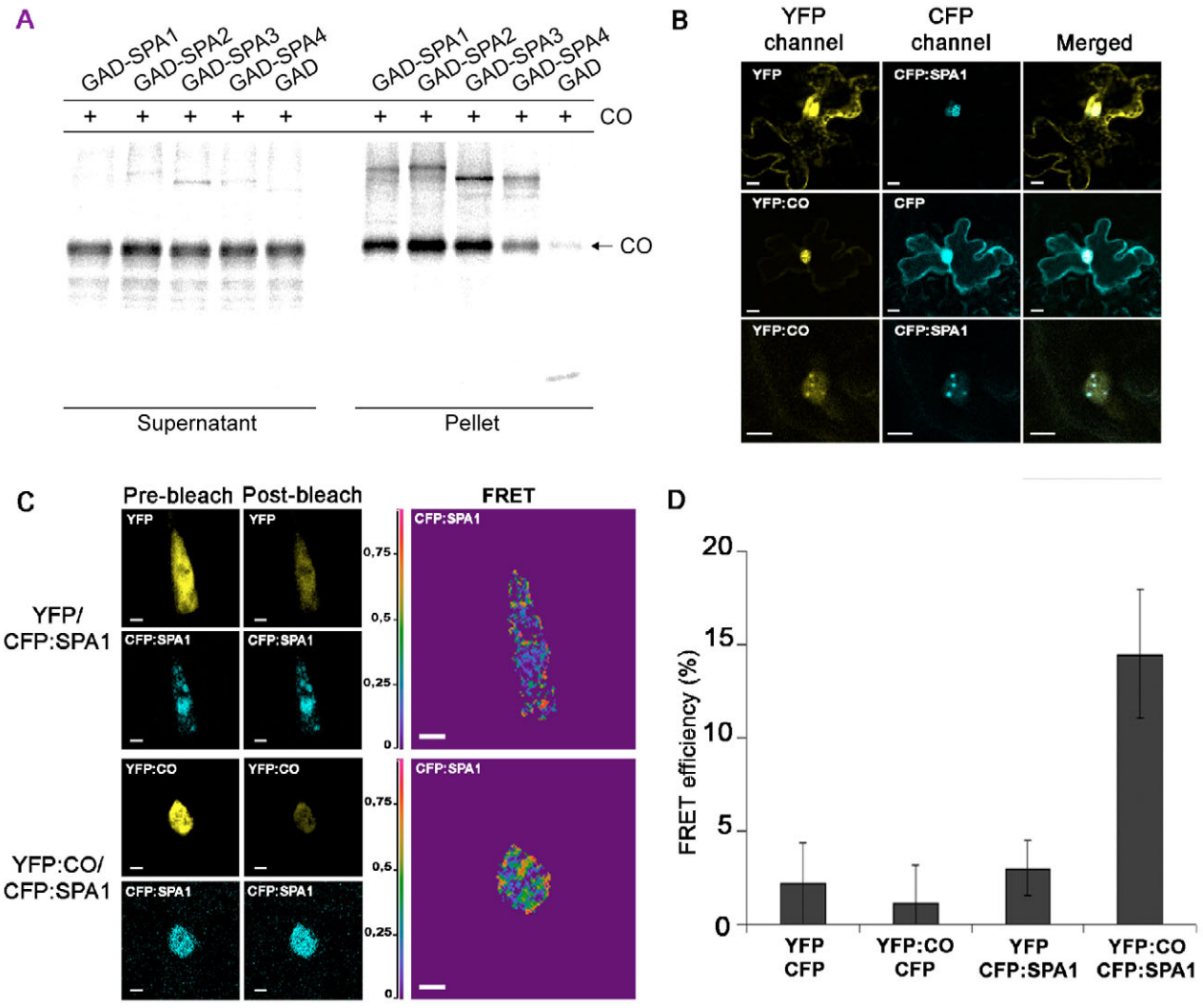

Fig. 5. SPA proteins physically interact with CO. (**A**) SPA proteins interact with CO in vitro. Recombinant ^{35}S-labelled CO was incubated with partially ^{35}S-labelled GAD-SPA proteins or GAD, respectively, and co-immunoprecipitated with anti-GAD antibodies. Supernatant fractions (1.6%) and 33.3% of the pellet fractions were resolved by SDS-PAGE and visualized by autoradiography using a phosphorimager. (**B**) Co-localization of CFP-SPA1 and YFP-CO in transiently transfected *Arabidopsis* leaf epidermal cells. Images were taken by confocal microscopy. Scale bar: 4 μm. (**C**) FRET microscopy by acceptor photobleaching. Fluorescence intensities of CFP-SPA1 and of YFP-CO or YFP constructs before and after acceptor photobleaching. Scale bar: 5 μm. (**D**) Comparison of FRET efficiency after acceptor photobleaching measured in nuclei. Data are mean±s.d. of 10-20 cells from three separate experiments.

DISCUSSION

SPA proteins represent a small four-member family that is required for suppression of photomorphogenesis in dark-grown *Arabidopsis* seedlings as well as for normal elongation growth of the adult plant (Laubinger et al., 2004). It has been shown previously that SPA proteins act in concert with another repressor of light signaling, the ubiquitin ligase COP1, which ubiquitinates light signaling activators in dark-grown seedlings (Hoecker and Quail, 2001; Laubinger et al., 2004; Saijo et al., 2003; Seo et al., 2003; Yang et al., 2005a). Here, we have demonstrated that SPA proteins are, moreover, required for photoperiodic flowering in the facultative LD plant *Arabidopsis*. More specifically, we show that SPA proteins physically interact with the floral promoter CO to inhibit flowering under non-inductive SD conditions. Taken together, our results suggest that SPA proteins regulate flowering time by controlling the stability of the CO protein.

SPA genes represent a new gene family required for photoperiodic flowering

Our phenotypic analysis has shown that mutations in the *SPA1* gene caused early flowering under SD conditions, but not under LD conditions. Mutating *SPA3* and *SPA4* in addition to *SPA1* further advanced flowering in SD to an extent that day-length control of flowering time was fully lost. Hence, *SPA1*, *SPA3* and *SPA4* are crucial for the normal delay of reproductive development that is observed in SD-grown *Arabidopsis* plants. Interestingly, *cop1* mutants also show de-repression of flowering in SD (McNellis et al., 1994). This suggests that SPA proteins and COP1 may act in concert to control photoperiodic flowering, as they do in the regulation of seedling skotomorphogenesis. The molecular mechanism of COP1-mediated control of flowering time has, however, thus far not been investigated.

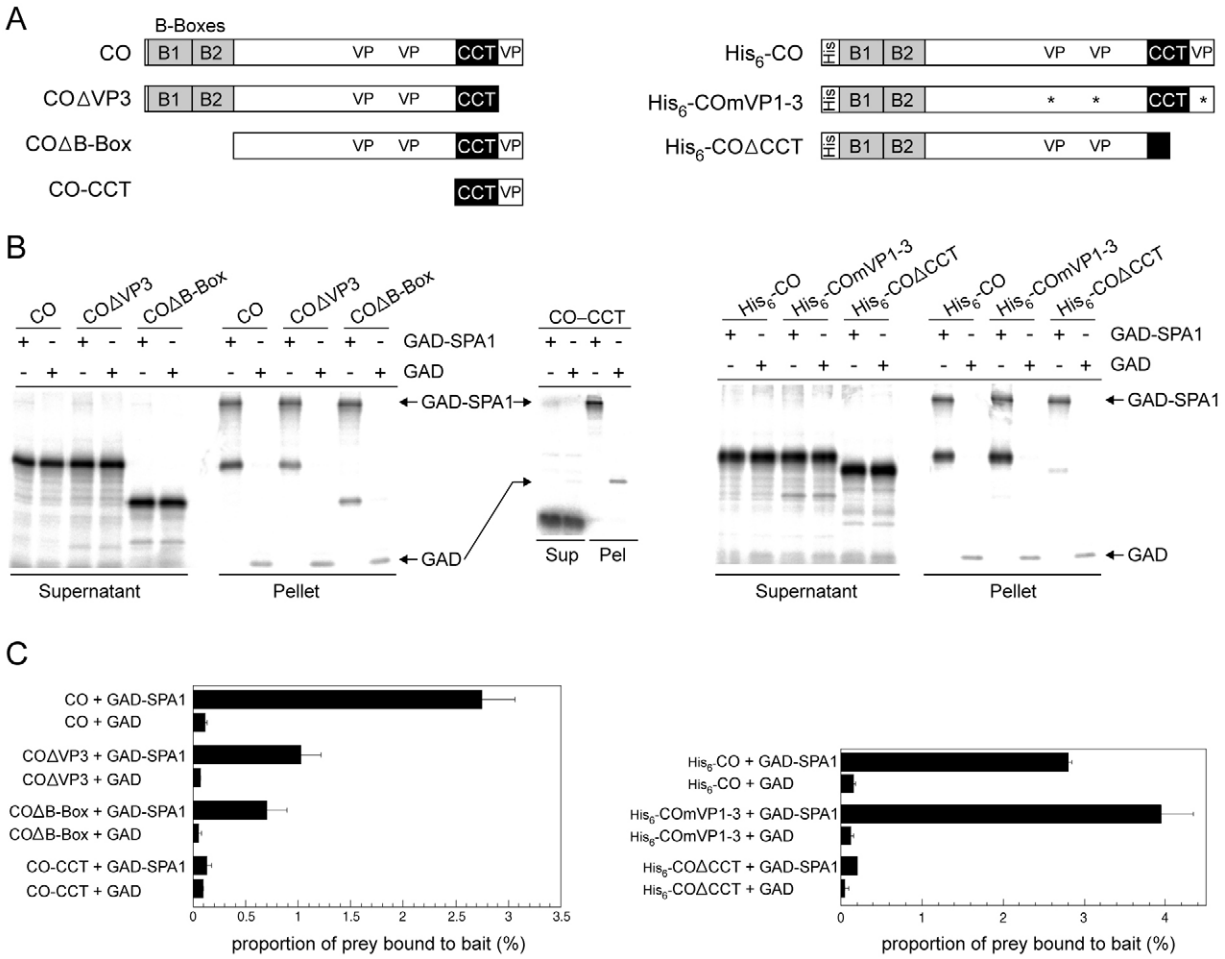

Fig. 6. The CCT domain of CO is necessary for the in vitro interaction with SPA1. (**A**) Schematic representation of the deletion-derivatives of CO or His_6-CO used in the in vitro binding assay. (**B**) Mapping of the SPA1-interacting domain of CO. Recombinant ^{35}S-labelled CO or indicated CO-deletion proteins were incubated with partially ^{35}S-labelled GAD-SPA1 or GAD, respectively, and co-immunoprecipitated with anti-GAD antibodies. Supernatant fractions (1.6%) and 33.3% of the pellet fractions were resolved by SDS-PAGE and visualized by autoradiography using a phosphorimager. (**C**) Quantification of the fractions of prey proteins that were co-immunoprecipitated by the indicated bait proteins GAD-SPA1 or GAD. Error bars depict the s.e.m. from two replicate experiments.

Although there is some functional redundancy among the four SPA genes, *SPA1* is clearly the predominant player in the regulation of flowering time. Only *spa1* mutants, but not *spa2*, *spa3* and *spa4* single mutants showed a defect in flowering time. Moreover, we found that *SPA1* is sufficient for normal photoperiodic flowering as triple mutants with defects in all SPA genes but *SPA1* flowered normally.

spa mutants flower early in SD due to strongly increased *FT* transcript accumulation

Early flowering of SD-grown *spa* mutants strongly correlated with increased *FT* transcript levels, with *spa1* mutants showing 70-fold higher and *spa1 spa3 spa4* mutants showing ~1000-fold higher *FT* mRNA abundance when compared with the wild type. This is consistent with previous findings showing that high-level *FT* expression in transgenic 35S::FT or 35S::CO plants led to early flowering even under non-inductive SD conditions (Samach et al., 2000; Kobayashi et al., 1999; Kardailsky et al., 1999).

The transcript levels of the flowering time gene *SOC1*, by contrast, were not dramatically altered in early-flowering *spa* mutant plants. This is in agreement with previous findings showing that *SOC1* seems to be more strongly regulated by the autonomous/vernalization pathway than by the photoperiod pathway. *SOC1* mRNA levels are only slightly reduced in the day length-insensitive *co* and *gi* mutant plants, while they are strongly affected by mutations in the vernalization/autonomous pathway (Lee et al., 2000; Samach et al., 2000). Conversely, *FT* transcript levels are very strongly dependent on *CO* (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Wigge et al., 2005). However, *SOC1* transcript levels are increased by overexpression of *FT*, suggesting that *SOC1* expression is activated by FT (Michaels et al., 2005; Moon et al., 2005; Yoo et al., 2005). In addition, *SOC1* expression in the shoot apical meristem is delayed in *ft* mutants (Searle et al., 2006). Spatial and quantitative relationships between *FT* and *SOC1* expression are not well understood. Hence, *FT*-induction in the SD-grown *spa* mutants might be too low to activate *SOC1*. Alternatively, *SOC1*-induction by FT might occur at developmental stages or in specific tissues that are not reflected under our experimental conditions.

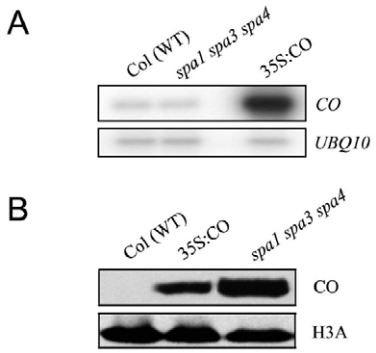

Fig. 7. CO protein levels are strongly increased in *spa1 spa3 spa4* triple mutants. (**A**) RT-PCR analysis of *CO* and *UBQ10* transcript levels in wild-type plants, *spa1 spa3 spa4* mutants and plants of a transgenic 35S-CO overexpressing line. (**B**) CO protein levels in nuclear extracts of wild-type plants, *spa1 spa3 spa4* mutants and plants of a transgenic 35S-CO overexpressing line. As a loading control, blots were reprobed with an antibody against histone H3A. Plants were grown in LD for 12 days and harvested at ZT16.

SPA1 interacts with CO to prevent CO-mediated induction of *FT* in SD

Our epistasis analysis demonstrates that SPA1 acts upstream of a key regulator of photoperiodic flowering, the putative transcription factor CO. Early flowering as well as the increase in *FT* transcript abundance was fully abolished in *spa1 co* double mutants. We can envision at least two not mutually exclusive possibilities on how SPA1 might control CO function: SPA1 might regulate the expression of *CO*. A change in the expression pattern of *CO* is known to alter photoperiodic flowering, as, for example, in the late-flowering mutants *fkf1* and *gi*, or the early-flowering mutant *toc1* (Imaizumi et al., 2005; Imaizumi et al., 2003; Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). Alternatively, SPA1 might regulate the stability and/or activity of the CO protein. Our results do not support the first model. The mutants *spa1* and *spa1 spa3 spa4* showed no difference in *CO* transcript abundance throughout a 24-hour time period (SD), when compared with the wild type, indicating that early flowering of these mutants was not caused by a change in the level or pattern of *CO* expression. We, therefore, favor the model that SPA1 regulates the CO protein. Indeed, we found that SPA1 and CO physically interact in vitro and in vivo. Thus, the mechanism of SPA1-mediated repression of *FT* in SD probably involves direct binding of SPA1 to CO.

Model on the function of SPA proteins in photoperiodic flowering

Genetic, functional and interaction analyses have shown that SPA proteins act together with the ubiquitin ligase COP1 to inhibit photomorphogenesis, probably by causing ubiquitination and subsequent degradation of light signaling activators (Laubinger et al., 2004; Saijo et al., 2003; Seo et al., 2003; Yang et al., 2005a). It is, therefore, plausible that SPA proteins might inhibit floral induction in SD by promoting the degradation of a positive regulator of flowering. Indeed, we found that *spa* mutations caused a strong increase in CO protein levels. Because the transcript levels of *CO* were unaffected by *spa* mutations, these results most probably reflect a reduction in CO protein degradation. Hence, our results support the idea that CO is subject to degradation via a mechanism that involves SPA1.

It is thus far unknown how SPA proteins regulate CO protein stability. CO was recently reported to be degraded under SD conditions (Valverde et al., 2004). In SD, *CO* transcripts accumulate primarily during the night phase, i.e. in darkness, and, therefore, synthesized CO protein is thought to be rapidly degraded by the proteasome (Valverde et al., 2004). Hence, we speculate that SPA proteins might be directly involved in this dark-dependent degradation of CO. This is consistent with previous evidence showing that SPA proteins function to suppress light signaling in darkness (Laubinger et al., 2004). In addition, our observation that SPA transcript levels rise during the night phase, i.e. when CO is degraded, supports this idea. Thus far, we could not investigate regulation and dynamics of SPA1-mediated CO degradation because CO is of too low abundance in SD-grown plants. However, our finding that CO protein abundance is higher in light-grown *spa* mutant plants when compared with the wild type indicates that CO is also degraded in the light and, thus, that light does not fully inhibit degradation of CO.

We also considered an alternative possibility that SPA proteins function in the light to inhibit the phyA-dependent stabilization of CO (Valverde et al., 2004). This mechanism is conceivable because mutations in *SPA1*, *SPA3* or *SPA4* cause a hyper-responsiveness of seedlings to light in a fashion that is fully dependent on a functional *PHYA* gene (Hoecker et al., 1998; Laubinger and Hoecker, 2003; Fittinghoff et al., 2006). This indicates that SPA proteins are especially important for normal phyA signaling in light-grown seedlings. However, our findings that early flowering of *spa1* mutants is independent of phyA and specific to SD are inconsistent with this model.

We thank Alexander Maier, Petra Fackendahl and Sabine Link for excellent technical assistance; Wilhelm Rogmann and his greenhouse staff for expert care of our plants; and members of the laboratory for helpful discussions. We are grateful to the Arabidopsis Biological Resource Center for providing mutant seed. This research was supported by the Deutsche Forschungsgemeinschaft (SFB 590, to U.H.) and by a core grant from the Max Planck Society (to G.C.).

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