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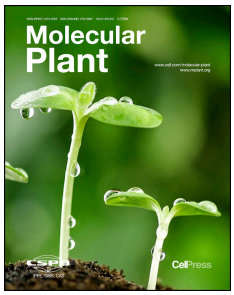

A *cop1 spa* mutant deficient in COP1 and SPA proteins reveals partial co-action of COP1 and SPA during Arabidopsis post-embryonic development and photomorphogenesis

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Letter to the editor:

A *cop1 spa* mutant deficient in COP1 and SPA proteins reveals partial co-action of COP1 and SPA during Arabidopsis post-embryonic development and photomorphogenesis

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Dear Editor,

The Arabidopsis CONSTITUTIVELY PHOTOMORPHOGENIC1/SUPPRESSOR OF PHYA-105 (COP1/SPA) complex is a key repressor of light signaling that inhibits light responses in darkness. It acts as an E3 ubiquitin ligase which ubiquitinates positively-acting light signaling intermediates, mainly transcription factors, thereby targeting them for proteolytic degradation by the 26S proteasome. In the light, photoreceptors directly interact with the COP1/SPA complex, leading to its inactivation which subsequently allows the target transcription factors to accumulate and to initiate vast reprogramming of gene expression (Huang et al., 2014).

Genetic and biochemical studies indicate that COP1 and SPA proteins act in concert to repress photomorphogenesis, i.e. as members of the COP1/SPA complex(es) (Laubinger et al., 2004; Yang and Wang, 2006; Zhu et al., 2008). However, a *spa cop1* null mutant lacking the whole COP1/SPA complex has never been described so far. Moreover, the phenotypes of *cop1* null mutants and *spa* quadruple mutants with mutations in all four *SPA* genes (*SPA1-SPA4*) are not identical, though this would be expected for a required co-action of COP1 and SPA proteins. *cop1* null mutants arrest growth at the seedling stage, whereas a *spa* quadruple mutant proceeds through development and produces seed, despite being very dwarfed (Laubinger et al., 2004; McNellis et al., 1994). However, the interpretation of SPA function in these *spa* mutants was hindered by the lack of null alleles. The *spa* quadruple mutant analyzed so far is not null for *SPA2* since the *spa2-1* allele produces and accumulates a truncated SPA2 protein lacking the C-terminal approx. 100 amino acids (Laubinger et al., 2004; Zhu et al., 2008). Also, *spa1-7* and *spa4-1* carry T-DNA insertions at the proximity of the 3´ end of the respective coding sequence, so that there is a possibility that truncated SPA1 and SPA4 proteins are produced. Hence, it cannot be excluded that the viability of this *spa* quadruple mutant is due to residual production of partially functional SPA proteins.

Here, we have isolated *spa* null mutant alleles and have generated a *spa* quadruple null mutant as well as two different types of *cop1 spa* quintuple mutants to address the following questions with respect to the degree of COP1/SPA co-action: 1) Are Arabidopsis plants which fail to produce any SPA proteins viable, i.e. does COP1 indeed have residual activity in the absence of SPAs? 2) Are COP1 and SPAs necessary for embryogenesis, i.e. do SPA proteins have residual activity in the absence of COP1 during embryogenesis? 3) Is the C-terminal WD-repeat domain truly essential for COP1/SPA function and can the SPA WD-repeat domains partially replace the functions of the WD-repeat domain of COP1?

By screening the MPIPZ T-DNA insertion collection, we identified genuine null alleles in *SPA2* and *SPA4* (*spa2-2*, *spa4-3*; Supplemental Figure 1). We crossed the new null alleles with the previously identified *spa1-100* and *spa3-1* null alleles to generate higher order *spa* mutants. The null *spa2-2* and *spa4-3* single mutants and derived double and triple *spa* null mutants exhibited seedling and adult phenotypes that were indistinguishable from those of the previously characterized multiple mutant allele combinations (Supplemental Figures 2, 3). The null *spa* quadruple mutant lacking all four SPA proteins, from now on referred to as *spaQn*, undergoes constitutive photomorphogenesis, is viable, fertile and able to complete its life cycle (Figure 1A,B), as was reported previously for the *spaQ* mutant which is not null for all four *SPA*s (Laubinger et al., 2004). This result confirms that plants lacking all SPA proteins are indeed viable which is in contrast with the seedling growth arrest observed in *cop1* null mutants (McNellis et al., 1994). Hence, we can now unambiguously conclude that COP1 alone, i.e. in the absence of SPA proteins, has residual activity that allows the plant to complete its life cycle. COP1 activity is nevertheless strongly enhanced by SPA proteins. *spaQn* mutants differed from *spaQ* mutants in that seedlings and plants appeared darker, suggesting higher anthocyanin content in *spaQn* than in *spaQ* plants (Figure 1A,B). Indeed, *spaQn* seedlings accumulated higher levels of anthocyanin than *spaQ* seedlings (Supplemental Figure 4A). Hence, the *spaQ* mutant has residual SPA activity, possibly due to the truncated SPA2-1 protein it produces.

Besides controlling seedling deetiolation and leaf expansion, the COP1/SPA complex is required to suppress flowering under non-inductive short day conditions (Laubinger et al., 2006; McNellis et al., 1994). Previous results indicated overlapping but also distinct functions of the four *SPA* genes in seedling growth and leaf expansion (Balcerowicz et al., 2011; Laubinger et al., 2004). The regulation of flowering time, however, has not yet been analyzed in this regard. Figure 1C shows that *SPA1* and *SPA4* are sufficient to strongly repress flowering in short day. Hence, *SPA1* and *SPA4* are the primary *SPA* genes responsible for photoperiodic flowering, while *SPA2* and *SPA3* provide only minor contributions in regulating the transition from vegetative to reproductive growth. *spaQn* mutants flowered very early in short day and long day and were, thus, fully insensitive to day length (Figure 1D).

COP1 function is thought to be specific to light signal transduction. On the other hand, *cop1* null mutants arrest growth at the seedling stage (McNellis et al., 1994), suggesting fundamental defects that may not solely be related to light signaling. Consistent with this idea, *cop1* mutants exhibit increased DNA damage, though this DNA damage can apparently be repaired prior to cell division (Dohmann et al., 2008). Human COP1 is also

involved in DNA damage-induced cell cycle block by controlling the stability of p53 (Dornan et al., 2004). However, Arabidopsis *cop1* null mutants proceed through embryogenesis, a process with complex and well-defined cell division patterns, suggesting that cell division is not fundamentally impaired in the absence of COP1. We therefore asked whether SPA proteins are at least partially active during embryogenesis and thus allow seed formation in the absence of COP1. To this end, we aimed to generate a homozygous *spaQn cop1-5* quintuple mutant which is fully devoid of both COP1 and SPAs. Indeed, homozygous *spaQn cop1-5* quintuple mutant seeds were identified in progeny of a selfed *spa123 (-/-) spa4-3* (+/-) *cop1-5* (+/-) plant. Hence, embryogenesis clearly does not require COP1/SPA function. In conclusion, fundamental cellular processes can proceed in the absence of COP1/SPA activity. Interestingly, light is required for growth of the shoot apex and leaf organ initiation. Hence, major disturbances specifically in meristem function of *cop1* and *cop1 spa* null mutants are likely responsible for the growth arrest at the seedling stage (Yoshida et al., 2011).

spaQn cop1-5 quintuple mutant seedlings had a shape very similar to that of *cop1-5* single mutants in darkness as well as in the light (Figure 1E). Both the quintuple mutant and the *cop1-5* mutant failed to develop beyond the seedling stage. In total, these results show that SPA proteins have no activity in the absence of COP1. The only detectable difference between *cop1-5* and the *spaQn cop1-5* quintuple mutant was a higher anthocyanin content in the quintuple mutant when compared to *cop1-5* or *spaQn* (Figure 1E, Supplemental Figure 4B). This suggests a possible COP1-independent function of SPA proteins in anthocyanin accumulation. However, since the *cop1-5* and the *spaQn* alleles were derived from different Arabidopsis accessions, we cannot exclude the possibility that these differences are due to the mixed genetic background in the quintuple null mutant.

In their C-termini, both COP1 and SPA carry a WD-repeat domain which mediates direct interactions with substrates as well as with DDB1 in the higher order CUL4-DDB1$^{COP1/SPA}$ E3 ubiquitin ligase (Chen et al., 2010; Huang et al., 2014). Generally, mutations in the respective WD repeat domain abolish COP1 and SPA1 function. Nevertheless, the *cop1-4* mutant which carries a premature STOP codon and therefore accumulates a truncated COP1 lacking all WD-repeats has only a partial loss-of-function phenotype (McNellis et al., 1994). This mutant is viable and has a plant size intermediate between those of the *spa* quadruple mutant and the wild type. Hence, the COP1-4 protein is partially functional despite the missing WD-repeat domain. To investigate whether the SPA proteins are responsible for the observed residual COP1-4 activity, we generated *cop1-4 spaQn* quintuple mutants. Figure 1F shows that this quintuple mutant had a "fusca"

phenotype that was more severe than those of the *cop1-4* and *spaQn* mutants. The *cop1-4 spaQn* quintuple mutant exhibited a seedling phenotype very similar to that of the *cop1-5* null mutant (Figure 1F) and, like *cop1-5*, failed to develop beyond the seedling stage (Supplemental Figure 5). This result indicates that the COP1-4 protein does not retain any activity in the absence of SPA proteins. We therefore conclude that the WD-repeat domains provided by the SPA proteins can at least partially substitute for the lack of the COP1 WD-repeat domain in the COP1-4 protein. The severe phenotype of the *cop1-4 spaQn* quintuple mutant further confirms that the WD repeats are essential for signaling activity of the COP1/SPA complex, i.e. a COP1-4 protein *per se* has no apparent activity.

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Figure 1 legend: Phenotypes of a *spa* quadruple null mutant (*spaQn*), a *spaQn cop1-5* quintuple null mutant deficient in all four SPAs and COP1, and a *spaQn cop1-4* quintuple mutant expressing only a truncated COP1 protein lacking the WD-repeat domain.

(A) *spaQn* quadruple mutants are viable, dwarfed plants. *spaQn* mutants carry null alleles at all four *SPA* loci. *spaQ* mutants carry previously described *spa* alleles that are - in part - not null. Plants were grown in long days for three weeks. The white bar indicates 1 cm. The inserts show 5x magnifications of *spaQ* and *spaQn* plants.

(B) *spaQn* seedlings undergo constitutive photomorphogenesis but appear more purple than *spaQ* mutants. *spaQ* and *spaQn* mutant seedlings were grown in darkness for 5 days. The black bar indicates 1 mm.

(C) *SPA1* and *SPA4* are the primary *SPA* genes controlling photoperiodic flowering. Flowering time was determined in *spa* triple mutants carrying *SPA* null alleles and in Col wild type (WT) grown in short day (SD).

(D) *spaQn* mutants flower constitutively early in short day (SD) and long day (LD).

(E) Quintuple *spaQn cop1-5* mutants devoid of COP1 and all four SPA proteins are capable of completing embryogenesis. Seedlings of the indicated homozygous genotypes were grown in darkness for 6 days or in white light (Wc, 25 µmol m^{-2} s^{-1}) for 4 days. The black bar indicates 1 mm.

(F) The WD-repeat domain of SPA proteins can partially replace a missing WD-repeat domain in COP1. *cop1-4* produces a truncated COP1 protein lacking the WD-repeat domain. Seedlings of the indicated homozygous genotypes were grown in darkness or white light (Wc, 25 µmol m^{-2} s^{-1}) for four days. The black bar indicates 1 mm.

Supplementary figure legends:

Supplementary Figure 1. T-DNA insertions in *spa2-2* and *spa4-3* cause null mutations

(A,B) *SPA2* (A) and *SPA4* (B) gene structure with schematic representation of T-DNA insertion sites in the new alleles (*spa2-2* and *spa4-3,* shown in bold) and previously used alleles (*spa2-1* and *spa4-1*). Black rectangles represent exons and lines denote introns.

(C,D) RT-PCR analysis of RNA isolated from wild-type (WT) and *spa2-2* (C) or *spa4-3* (D) mutant seedlings using primers flanking the respective T-DNA insertion site. *SPA3*-specific primers were used as a positive control.

(E,F) RNA blot analysis of RNA isolated from wild-type (WT) and *spa2* (E) or *spa4* (F) mutant seedlings. RNA blots were hybridized with a *SPA2*- (E) or *SPA4*-specific probe (F). As a control for equal loading, blots were hybridized with an *18S* rRNA-specific probe.

Supplementary Figure 2. Seedling photomorphogenesis in *spa2-2* and *spa4-3* single mutants is similar to that in mutants with previously identified alleles (*spa2-1*, *spa4-1*).

(A-F) Hypocotyl length of the indicated genotypes grown in continuous far-red (FRc) **(A,D)**, red (Rc) **(B,E)** or blue (Bc) light **(C,F)** of various fluence rates for 4 days. Error bars indicate the SEM.

Supplementary Figure 3: *spa* null double and triple mutants have a similar phenotype as the previously described *spa* mutants

(A,B) Visual phenotype of the indicated genotypes grown in darkness (A) or FRc (0.4 µmol m^{-2} s^{-1}) (B) for 4 days. For each genotype 2 different allele combinations are shown. Mutants

containing previously available alleles (left): *spa1-7*, *spa2-1*, *spa3-1*, *spa4-1*. *spa* null multiple mutants containing the null alleles (right): *spa1-100*, *spa2-2*, *spa3-1*, *spa4-3*. Scale bar: 10 mm.

(C,D) Hypocotyl length of the indicated genotypes grown as in A, B. Error bars indicate the SEM.

(E) Visual phenotype of three-week-old plants grown in long day (LD). Genotypes are as in A,B.

Supplementary Figure 4: Anthocyanin content in *spaQn* null mutants and *spaQn cop1-5* quintuple mutants

(A) Anthocyanin content per 10 seedlings in *spaQ* and *spaQn* mutant seedlings grown in darkness for 5 days. The error bars show the SEM (n=3). Asterisks indicate significant differences (Student´s t-test: *** P< 0.001).

(B) Anthocyanin content in mature embryos of the indicated genotypes. Mature embryos were manually dissected from the maternal seed coat. Anthocyanin content is shown per 15 mature embryos. Error bars represent SEM (n=3). Asterisks indicate significant differences (Student´s t-test: ***P< 0.001 when compared to either parent).

Supplementary Figure 5: *spaQn cop1-4* quintuple mutants arrest growth at the seedling stage

Phenotype of the indicated genotypes grown in white light (Wc, 25 µmol m^{-2} s^{-1}) for 15 days. The black bar indicates 1 mm.

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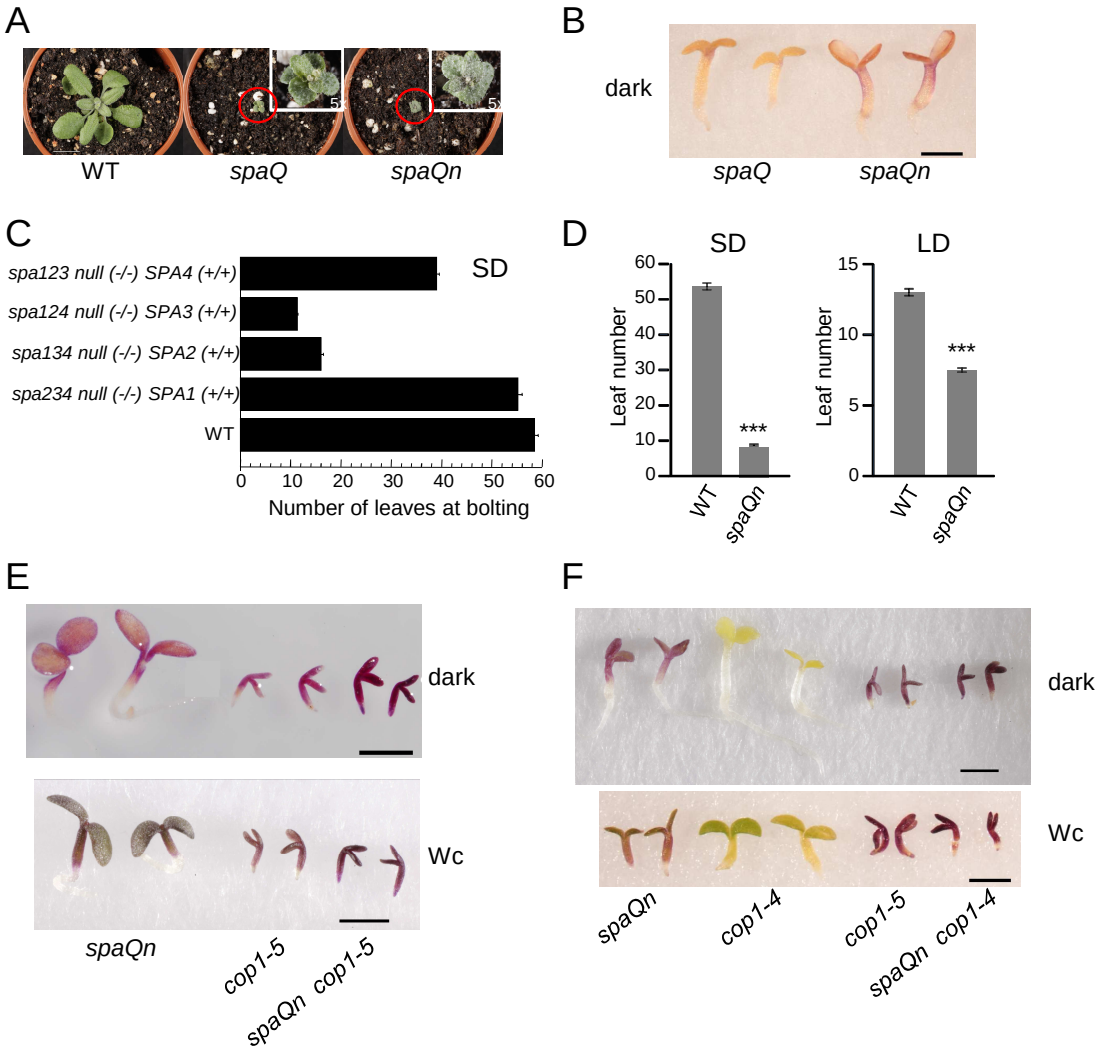

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