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Widespread Role for the Flowering-Time Regulators FCA and FPA in RNA-Mediated Chromatin Silencing

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therefore ran follow-up probe trials to determine whether the chimpanzees were capable of attending to the amount of food available to the partner. Subjects were tested alone, and they had to look into the distal food dishes to correctly choose the tray that would yield the largest payoff from the partner's position before going through the open door to the adjacent cage to get it. They chose correctly at greater than chance levels, demonstrating that they would have been capable of seeing payoffs to the partner (*27*). Second, in inhibition probe trials, we found that subjects could inhibit pulling the rod when it led to no food gain about 64% of the time, about the same rate of pulling as in the 10/0 condition, suggesting that some of the failure to reject zero offers was due, at least some of the time, to an inability to inhibit a natural tendency to pull. Third, in discrimination probe trials, responders could distinguish between all offers available to them (fig. S2), and proposers could do so for all but 10/0 versus 8/2 (fig. S1) (*31*), demonstrating that subjects were able to make maximizing choices.

Our subjects were from a single social group, they did not interact anonymously, and they played both roles in the game. However, anonymous one-shot games are used in experiments with humans to decrease the likelihood of making fair offers or accepting unfair offers (*32*, *33*), and so if anything, our experimental design should have been skewed in favor of finding fairness sensitivity. The fact that chimpanzees in this study did not punish other individuals for making unfair offers may be in part a reflection of the fact that active food sharing is rare in this species (*34*) and may also be because they were unwilling to pay a cost to punish.

We gave chimpanzees the most widely recognized test for a sensitivity to fairness, the ultimatum game, and found that they did not systematically make fair offers to conspecifics, nor did they systematically refuse to accept unfair offers from conspecifics even though they could discriminate between the quantities available to themselves and their partners. It thus would seem that in this context, one of humans' closest living relatives behaves according to traditional economic models of self-interest, unlike humans, and that this species does not share the human sensitivity to fairness.

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Widespread Role for the Flowering-Time Regulators FCA and FPA in RNA-Mediated Chromatin Silencing

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The RRM-domain proteins FCA and FPA have previously been characterized as flowering-time regulators in *Arabidopsis*. We show that they are required for RNA-mediated chromatin silencing of a range of loci in the genome. At some target loci, FCA and FPA promote asymmetric DNA methylation, whereas at others they function in parallel to DNA methylation. Female gametophytic development and early embryonic development are particularly susceptible to malfunctions in FCA and FPA. We propose that *FCA* and *FPA* regulate chromatin silencing of single and low-copy genes and interact in a locus-dependent manner with the canonical small interfering RNA–directed DNA methylation pathway to regulate common targets.

Heterochromatin in many organisms is characterized by extensive DNA methylation and histone modifications (*1*). Plants display cytosine methylation in CG, CNG (N = any nucleotide), and CHH (H = A, C, or T) sequence contexts. In *Arabidopsis*, small interfering RNAs (siRNAs) are involved in localizing and maintaining these chromatin modifications in processes requiring *RNA-DEPENDENT RNA POLYMERASE2* (*RDR2*), *DICER-LIKE3* (*DCL3*), *ARGONAUTE4* (*AGO4*), and the two RNA polymerase IV isoforms, Pol IVa and b (*2*–*9*).

To identify further components required for siRNA-mediated chromatin silencing, we used a reporter system in which the *Arabidopsis* phytoene desaturase (*PDS*) gene is silenced in response to a homologous inverted repeat (*SUC-PDS*) (*10*). Two mutants that partially suppressed the silencing of *PDS* (Fig. 1, A, B, C, and E) showed late flowering that was reversible by vernalization. The silencing and flowering phenotypes cosegregated, and the mutations mapped to chromosomes 2 and 4. The flowering phenotype suggested involvement of *FPA* and *FCA*, two members of the autonomous pathway (*11*), mapping to those genomic regions. Sequencing revealed a premature termination codon in *FPA* (Trp^{98}*, G to A, *fpa-8*) and *FCA* (Gln^{537}*, C to T, *fca-11*). The flowering defect was confirmed by complementation analysis with previously known flowering mutants (*fca-9*, *fpa-7*, and *fve-3*; Fig. 1F), which also showed *PDS* silencing (fig. S1). Thus, *FCA* and *FPA* are required

for efficient *PDS* silencing in the presence of *SUC-PDS*.

FCA and FPA contain multiple RNA recognition motif (RRM) RNA binding domains (*12*, *13*), which are known to bind single-stranded RNA, but share no other sequence homology. FCA negatively regulates its own expression through alternative polyadenylation site usage (*14*). Late flowering in *fca* and *fpa* is due to overexpression of the major repressor of flowering in *Arabidopsis*, *FLOWERING LOCUS C* (*FLC*) (*11*). *FCA* and *FPA* do not appear to regulate *PDS* in the absence of the silencing trigger, which suggests that the presence of the transgene makes the endogenous *PDS* a target of *FCA* and *FPA*.

Because FCA and FPA both contain RRM domains, we hypothesized that they act partially redundantly; consistent with this, an *fca-11 fpa-8* double mutant showed no *PDS* silencing (Fig. 1D). Components of the siRNA chromatin-silencing pathway (the Pol IVa largest subunit NRPD1a and RDR2) also suppress *PDS* silencing completely (*10*). Both *nrpd1a-5* and *fca-11 fpa-8* double mutants showed reduced *SUC-PDS* and higher *PDS* mRNA levels (Fig. 1G and fig. S2) (*10*). *PDS* siRNA levels were reduced in *rdr2-5*, *nrpd1a-5*, and *fca-11 fpa-8* mutants, but not in *fca-11* or *fpa-8* single mutants (Fig. 1H).

Bisulfite sequencing was used to investigate whether *PDS* silencing corresponded to DNA methylation at the endogenous *PDS* locus. We found non-CG methylation (CNG and CHH) at the endogenous *PDS* locus in a region complementary to the hairpin in *SUC-PDS* leaves, but not in wild-type leaves (Fig. 1I and table S1). CG sites were highly methylated in both the wild type and mutants. In *nrpd1a-5* and *fca-11 fpa-8* mutants, loss of siRNA coincided with loss of asymmetric DNA methylation (Fig. 1I and table S1). CHH methylation was also compromised in *fca-11* and *fpa-8* single mutants, although *PDS* siRNA levels were unaffected (Fig. 1, H and I). These results suggest a dual role for *FCA* and *FPA*: (i) They act together with *NRPD1a* and *RDR2* and redundantly with each other to amplify siRNAs derived from the transgene locus; (ii) they act in the perception and interpretation of the silencing signal at the target locus. Mutants in two other members of the autonomous pathway—the MSI1 homolog *FVE* and the putative histone demethylase *FLD* (*15*, *16*)—also suppressed *PDS* silencing (fig. S1), which indicates that multiple components of the autonomous pathway are involved in this process.

Transposons, retroelements, and intergenic transcripts are endogenous targets of chromatin-silencing pathways (*5*–*8*, *17*). Expression of the *AtSN1* retroelement and the *AtMu1* DNA transposon were also controlled by FCA and FPA (Fig. 2A). *AtSN1* was reactivated very strongly in *fpa-8*, *fca-11 fpa-8*, and *nrpd1a-5* mutant seedlings, but not in *fca-11*. In contrast, *AtMu1* was slightly derepressed in *fca-11* and *fpa-8* single mutants and more strongly in *fca-11 fpa-8*. *AtMu1* reactivation in *fca-11 fpa-8* was similar to that in *nrpd1a-5*. An intergenic transcript flanked by a solo long terminal repeat (LTR), *IG/LINE*, was also up-regulated in *fca-11 fpa-8*, albeit to a lesser extent than in *nrpd1a-5* (Fig. 2A). Together, these findings indicate that *FCA* and *FPA* have a widespread role in the regulation of endogenous loci known to be silenced at the level of transcription and dependent on siRNA.

We next investigated whether this transcriptional reactivation correlated with loss of corresponding siRNA. *AtSN1* and *AtMu1* siRNAs were detected at wild-type levels in *fca-9*, *fpa-7*, and *fca-9 fpa-7*, but were absent from *nrpd1a-3* mutant seedlings (Fig. 2B). Corresponding results were obtained for other siRNAs. Thus, despite their role in the amplification of *PDS* siRNA, *FCA* and *FPA* do not generally act in NRPD1a-dependent siRNA production. There was no change in DNA methylation at the *AtSN1* locus in *fca fpa* (Fig. 2C, fig. S3, A and B, and table S2). However, bisulfite sequencing indicated a reduction of ~50% in asymmetric (CHH) DNA methylation at *AtMu1* in *fca fpa*, whereas CG and CNG methylation were not affected (Fig. 2C, fig. S3B, and table S2). Likewise, asymmetric DNA methylation at the solo LTR was reduced (fig. S3C). Maintenance of asymmetric DNA methylation requires the continued presence of the trigger, whereas symmetric DNA methylation can be maintained through cell divisions in the absence of the trigger. Silencing at these loci is also associated with changed histone tail modifications such as increased H3 K9 dimethylation and reduced H3 K4 dimethylation (*5*, *8*, *17*). Using chromatin immunoprecipitation, we did not find any pronounced alteration in these marks in *fca-9 fpa-7*.

Heterochromatic loci are targeted by multiple silencing pathways, and their contribution at individual loci differs considerably (*18*–*20*). This is corroborated by our finding that silencing of *AtSN1*, *AtMu1*, *IG/LINE*, and *PDS* in the presence of *SUC-PDS* differentially requires

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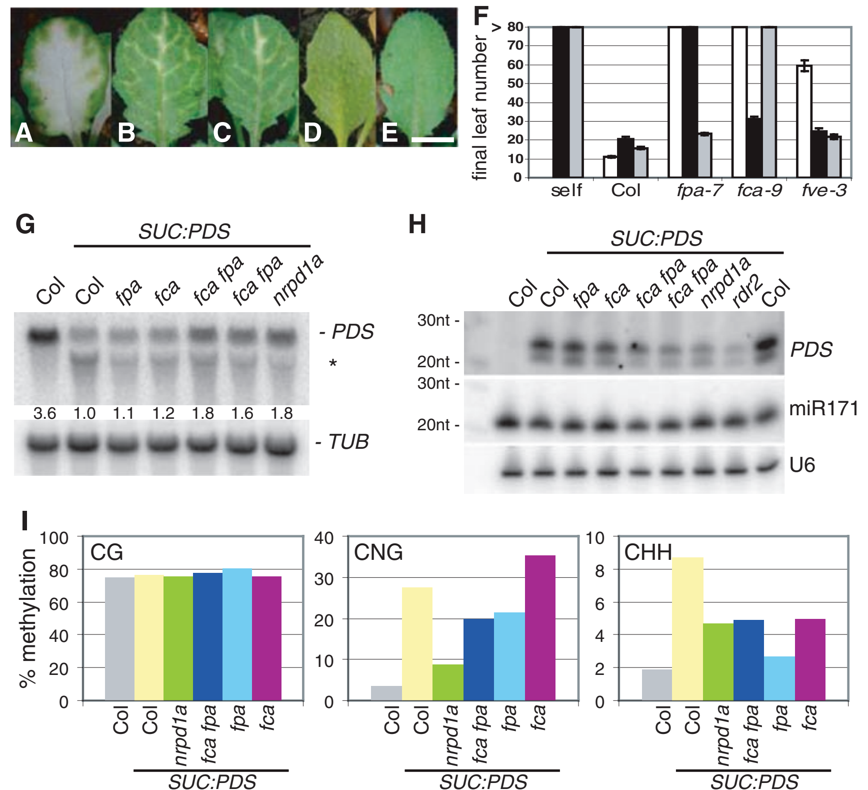

Fig. 1. *FCA* and *FPA* suppress *SUC-PDS*–induced silencing of *PDS*. (**A** to **E**) Leaf phenotypes in *SUC-PDS* background grown in long days. (A) Col, (B) *fpa-8*, (C) *fca-11*, (D) *fca-11 fpa-8*, (E) no transgene. Scale bar, 5 mm. (**F**) Complementation analysis: average flowering time (±SEM) of F_1 progeny of crosses between the indicated mutations (white, selfed) and *fpa-8* (black) or *fca-11* (gray). (**G**) RNA gel blot analysis of *PDS* mRNA detecting endogenous *PDS* (*PDS*) and *SUC-PDS* mRNA (*). Numbers indicate relative expression of *PDS* averaged over two experiments. (**H**) RNA gel blot analysis of *SUC-PDS* siRNA. (**I**) Cytosine methylation at the endogenous *PDS* locus assayed by bisulfite sequencing.

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FCA and *FPA*. *PDS* silencing is associated with target DNA methylation and siRNA production through mechanisms that are dependent on both the siRNA chromatin-silencing pathway and *fca fpa*. Derepression of *AtMu1* and *IG/LINE* in *fca fpa* mutants coincides with loss of DNA methylation but not siRNAs, whereas both are lost in mutants of the siRNA chromatin-silencing pathway. Despite much stronger reactivation of *AtSN1* in *fca fpa*, neither DNA methylation nor siRNA accumulation was affected. Our findings are consistent with the idea that transcription can be reactivated in the presence of DNA methylation, as was established for the *morpheus' molecule 1* (*mom1*) mutation (*19*, *21*). Despite this similarity, it seems unlikely that *FCA* and *FPA* generally act together with *MOM1*, because *AtSN1* and *AtMu1* are not misregulated in *mom1* (*22*).

To investigate how *FCA* and *FPA* relate to the chromatin siRNA amplification pathway including *Pol IVa*, *RDR2*, and *DCL3*, we analyzed the release of silencing in double mutants (Fig. 2D). All double mutants showed much higher reactivation of *AtSN1* and *AtMu1* than any of the single mutants, which suggests that *FCA* and *FPA* do not act downstream of the siRNA amplification pathway, but rather in parallel. Similarly, transposon reactivation was greatly enhanced in *fve nrpd1a* double mutants relative to either of the single mutants (fig. S3D). Strikingly, although *FCA* is dispensable for *AtSN1* silencing in the wild type, the loss of *FCA* in *nrpd1a*, *rdr2*, or *dcl3* mutant backgrounds greatly enhanced the release of *AtSN1* silencing.

Our findings predict that perturbation of DNA methylation in *fca fpa* mutants will affect reactivation of target loci differently. At *AtSN1*, where the effect of *FCA* and *FPA* is uncoupled from DNA methylation, enhanced loss of silencing in the presence of the DNA methylation inhibitor 5-aza-deoxycytidine (aza-dC) would be expected. Conversely, at *AtMu1*, where *fca fpa* mutants show reduced DNA methylation, the additional effect of the inhibitor would be small. Our results (Fig. 3A and table S3) are consistent with this prediction, because *fca-9 fpa-7* mutants were more sensitive than the wild type to aza-dC with respect to *AtSN1* reactivation, but less sensitive than the wild type with respect to *AtMu1* reactivation. Also, development of *fca-9 fpa-7* seedlings was strongly perturbed when exposed to aza-dC at concentrations where development of wild-type or *fca-9* seedlings was not abnormal and development of *fpa-7* seedlings was only very slightly abnormal (Fig. 3B and table S4) (*23*).

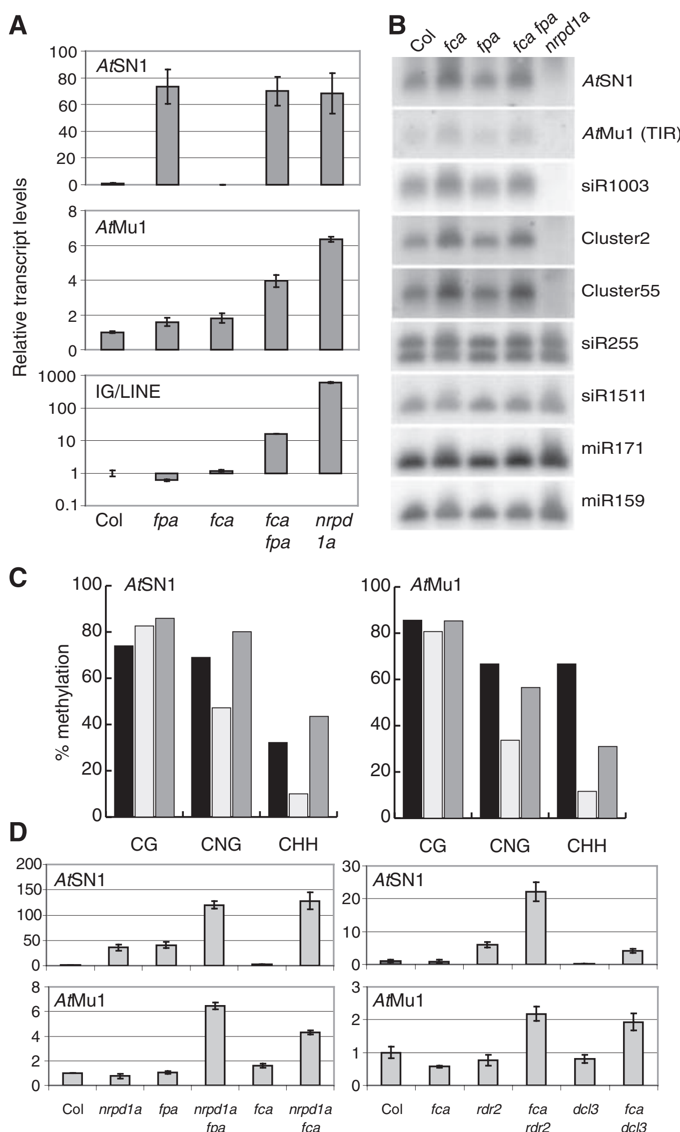

Fig. 2. Reactivation of *AtSN1*, *AtMu1*, and *IG/LINE* in seedlings. (**A**) Quantitative reverse transcription polymerase chain reaction (RT-PCR) on Col, *fpa-8*, *fca-11*, *fca-11 fpa-8*, and *nrpd1a-5*. (**B**) RNA gel blot analysis of trans-acting siRNAs (siR255, siR1511), microRNAs (miR159, miR171), or siRNAs (all other) on Col, *fca-9*, *fpa-7*, *fca-9 fpa-7*, and *nrpd1a-3*. (**C**) Cytosine methylation for Col (black), *nrpd1a-3* (light gray), and *fca-9 fpa-7* (dark gray). (**D**) Quantitative RT-PCR (left: Col, *nrpd1a-3*, *fpa-8*, *nrpd1a-3 fpa-8*, *fca-11*, and *nrpd1a-3 fca-11*; right: Col, *fca-9*, *rdr2-1*, *fca-9 rdr2-1*, *dcl3-1*, and *fca-9 dcl3-1*). *nrpd1a-3* is a weaker allele than *nrpd1a-5* with respect to *AtMu1* reactivation; error bars indicate SD.

fca fpa double mutant plants are late flowering but otherwise largely normal. However, closer examination of *fca-11 fpa-8* siliques revealed that ~20% of developing seeds aborted and ~70% of ovules did not initiate development (fig. S4A and Table 1). When pollinating double mutants with wild-type pollen, no seeds aborted, but the high proportion of undeveloped seeds persisted; this finding suggested that the embryonic lethality was zygotic, whereas the undeveloped seed phenotype was caused by the genotype of the mother plant. When *fca/fca FPA/fpa* ovules were pollinated with wild-type pollen, 34% of seeds appeared undeveloped (Table 1). Microscopic examination of mature ovules did not reveal any abnormalities (fig. S4, B and C), which suggests that the genotype of the female gametophyte determined the undeveloped seed phenotype. Thus, (female) gametophytic and early embryonic development is extremely sensitive to loss of *FCA* and *FPA*. Once these stages are passed successfully, development can proceed largely independently of *FCA* and *FPA*. Whether misregulation of a few key genes or more global genome misorganization causes these defects remains to be investigated.

We propose that the increased transcript levels measured for the targets in *fca fpa* reflect transcriptional reactivation rather than increased cytoplasmic RNA stability. This is supported by the subcellular localization of FPA and FCA: A fully complementing FPA–yellow fluorescent protein (YFP) fusion protein localized to the nucleus (Fig. 3C and fig. S5); FCA is a nuclear protein that interacts with the SWI/SNF chromatin remodeler SWI3B (*14*, *24*). Both proteins associate with the chromatin of their target genes: The FPA-YFP fusion protein localized to the chromatin of *AtMu1* and *FLC* (Fig. 3D); FCA localized to *FLC* chromatin (*25*). Lastly, using an established assay for transcriptional activity (*26*), *FLC* and *AtMu1* unspliced (nascent) transcripts were up-regulated in all backgrounds that caused up-regulation of the spliced transcript, and both unspliced and spliced transcripts were increased similarly (Fig. 3, E and F). Together, these data all indicate that silencing does not occur posttranscriptionally but rather cotranscriptionally before any processing occurs.

Taken together, our results show that the nuclear proteins FCA and FPA have a much more widespread role in development and gene silencing than previously anticipated. We propose a model in which FCA and FPA cotran-

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scriptionally recognize aberrant RNA and mark it for silencing (fig. S6). A nascent RNA may be made aberrant by the presence of low levels of complementary siRNAs or misconducted processing events. FCA and FPA would then facilitate silencing by recruiting or stabilizing effector complexes. Although the common result of FCA and FPA action is silencing of a target locus, the identity of these effector complexes presumably varies with the contribution of different pathways at individual loci, thus leading to somewhat different silencing signatures. Whereas the majority of functionally characterized RRM-domain proteins act in posttranscriptional RNA processing (*27*), FCA and FPA appear to integrate the state of the nascent RNA with transcription. That this might be a novel function of some RRM-domain proteins is supported by two other reports. The yeast Set1 histone methyltransferase has an RRM domain thought to bind nascent RNA and thereby regulate the methyltransferase activity (*28*). Furthermore, three RRM-domain proteins are required for transcriptional silencing in *Caenorhabditis elegans* cosuppression (*26*).

Although the canonical siRNA-directed chromatin-silencing pathway has been described for repetitive loci, FCA and FPA silence mainly single-copy loci and do not affect silencing of the highly repetitive 5S loci (fig. S7). At a subset of targets, however, these pathways clearly interact. The canonical chromatin-silencing/siRNA amplification pathway involves amplification of siRNAs and shuttling of silencing information between the locus and a nucleolar RNA processing center (*29*, *30*), thereby silencing any sufficiently homologous locus in the genome. In contrast, FCA and FPA may bypass the siRNA amplification step, thereby restricting it to acting in cis. Unraveling the interactions between the different pathways will ultimately enable us to understand what properties in a target commit it to being silenced in a particular way.

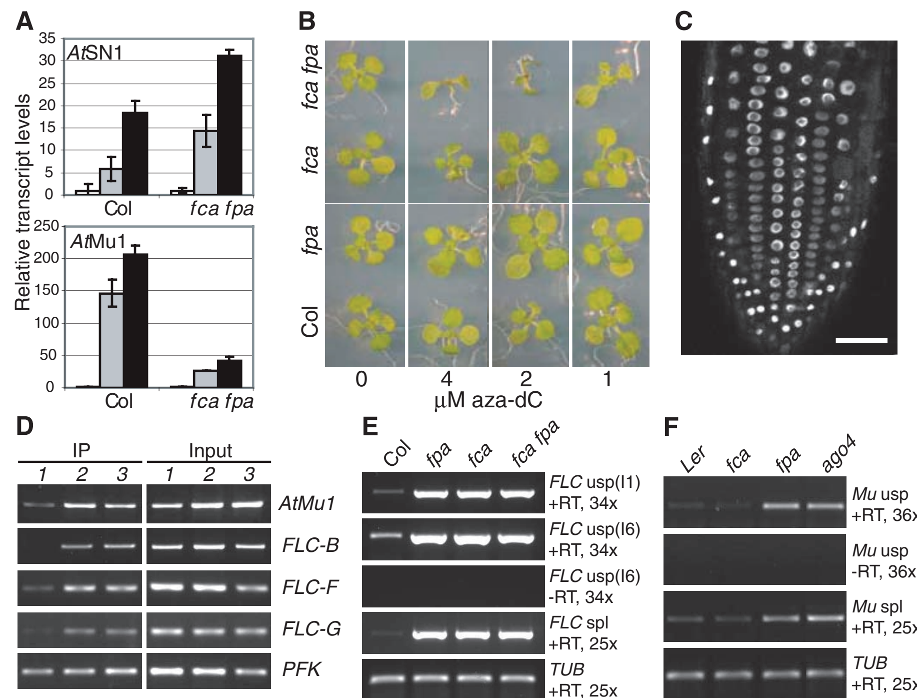

Fig. 3. (**A** and **B**) Aza-dC treatment. (A) Quantitative RT-PCR on Col and *fca-9 fpa-7* seedlings grown on aza-dC (white, mock; gray, 2 μM; black, 4 μM) normalized to the expression level after mock treatment (±SD). (B) Seedlings (Col, *fpa-7*, *fca-9*, and *fca-9 fpa-7*) grown for 14 days on aza-dC. (**C**) An FPA-YFP fusion protein localizes to the nucleus of transgenic *Arabidopsis* seedling roots. Scale bar, 50 μm. (**D**) Chromatin immunoprecipitation from two independent FPA-YFP lines. Lane 1, Col; lane 2, FPA-YFP line 2; lane 3, FPA-YFP line 5. (**E** and **F**) RT-PCR assaying spliced and unspliced transcripts of *FLC* and *AtMu1*.

Table 1. Percentage of aborted and undeveloped seed in *fca-11 fpa-8* mutant lines.

Parental genotype (female × male)	Healthy (%)	Aborted (%)	Undeveloped (%)	*n*
Col (SUC-PDS) selfed	100.0	0.0	0.0	206
fca-11 fpa-8 selfed	21.3	4.7	74.0	572
Col (SUC-PDS) × *fca-11 fpa-8*	82.7	0.0	17.3	572
fca-11 fpa-8 × Col (SUC-PDS)	30.9	0.0	69.1	375
fca-11 selfed	99.1	0.0	0.9	559
fpa-8 selfed	75.0	0.0	25.0	464
fca-11/fca-11 FPA/fpa-8 × Col	63.9	1.6	34.4	244

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References

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