Altered interactions within FY/AtCPSF complexes required for *Arabidopsis* FCA-mediated chromatin silencing

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The role of RNA metabolism in chromatin silencing is now widely recognized. We have studied the *Arabidopsis* RNA-binding protein FCA that down-regulates an endogenous floral repressor gene through a chromatin mechanism involving histone demethylase activity. This mechanism needs FCA to interact with an RNA 3′ processing/polyadenylation factor (FY/Pfs2p), but the subsequent events leading to chromatin changes are unknown. Here, we show that this FCA–FY interaction is required for general chromatin silencing roles where hairpin transgenes induce DNA methylation of an endogenous gene. We also show 2 conserved RNA processing factors, AtCPSF100 and AtCPSF160, but not FCA, are stably associated with FY in vivo and form a range of different-sized complexes. A hypomorphic *fy* allele producing a shorter protein, able to provide some FY functions but unable to interact with FCA, reduces abundance of some of the larger MW complexes. Suppressor mutants, which specifically disrupt the FY motif through which FCA interacts, also lacked these larger complexes. Our data support a model whereby FCA, perhaps after recognition of a specific RNA feature, transiently interacts with FY, an integral component of the canonical RNA 3′ processing machinery, changing the interactions of the different RNA processing components. These altered interactions would appear to be a necessary step in this RNA-mediated chromatin silencing.

flowering | gene silencing | RNA processing

Noncoding RNAs are now thought to play major roles in directing chromatin silencing (1). We have pursued the mechanistic basis of RNA-mediated silencing of the *Arabidopsis* floral regulator *FLOWERING LOCUS C* (*FLC*) by the autonomous pathway (2–4). This pathway has a widespread role in chromatin silencing in the *Arabidopsis* genome (5) and involves both RNA-binding (FCA, FPA, FLK)/RNA 3′ processing proteins (FY) and putative chromatin regulators (FVE, FLD and LD) (6). The RRM proteins FCA and FPA at least partially require one of the chromatin regulators, FVE–an MSI homologue—or FLD, an LSD1 homologue) to effect silencing; however, the requirements for specific chromatin regulators differ at different loci (7). Our current view is that this pathway functions as part of a transcriptome surveillance mechanism, transcriptionally down-regulating loci producing ''aberrant'' transcripts.

The RNA-binding protein FCA was used in affinity purification experiments to clone FY, the *Arabidopsis* homologue of the essential yeast RNA 3′ processing factor Pfs2p (8). *fy* null alleles were found to be embryo lethal (9); however, the initial *fy* alleles (*fy-1*, *fy-2*) were viable, just late-flowering because of production of a truncated peptide lacking the FCA interaction domain (9). These results suggested that FY might be a component of the *Arabidopsis* core RNA processing machinery, with the flowering-specific defects resulting from the loss of the FCA–FY interaction. FCA would then regulate the recruitment of specific transcripts to the core processing machinery. This classic post-transcriptional function was recently found to be an oversimplification. Suppressor mutagenesis experiments revealed that FLD, the homologue of the human lysine specific histone demethylase LSD1, is required for FCA to down-regulate *FLC* (10). FCA was found to regulate *FLC* transcriptionally via histone demethylation, and some of the RNAi machinery appeared to play a role in this mechanism (10).

Other RNA processing components from *Arabidopsis*–a CstF-64-like protein–symplekin/PTA1, AtCPSF100, and the splicing factor Prp2 have also been shown to play a role in gene silencing (11), and splicing factors have been found to facilitate RNAi-directed silencing in fission yeast (12). In mammals, the functionally analogous protein to Pfs2p, a 7-WD repeat protein that links different 3′-end cleavage and polyadenylation processing complexes (13, 14), is considered to be CstF-50. Interestingly, plant genomes contain Pfs2p and CstF-50 homologues (8, 15). Other mammalian RNA 3′ processing factors are cleavage and polyadenylation specificity factors (CPSF) CPSF160, CPSF100, CPSF73, and CPSF30; cleavage stimulation factors (CstF); cleavage factors (CF); poly(A) polymerase (PAP); and poly(A)-binding protein (14). Although CstF and CF are required for the cleavage reaction only, PAP and CPSF are involved in both cleavage and poly(A) tail addition.

We have pursued how the *Arabidopsis* RNA-binding protein FCA functions through FY, an RNA processing component, to trigger chromatin silencing. Here, we analyze the role of FY in FCA-mediated DNA methylation and address how these proteins interact in vivo, both with each other and with the RNA 3′ processing machinery.

Results

FY Regulates *FLC* Expression Cotranscriptionally and Is Required for RNA-Mediated Silencing Induced by a Hairpin. FCA has been shown to have a widespread role in transgene-induced and transposon silencing in the *Arabidopsis* genome, potentially detecting aberrant transcripts and silencing the loci through DNA methylation (5). FCA has also been shown to transcriptionally silence *FLC* through a histone demethylase activity conferred by FLD (10). Because FY is required for FCA-mediated down-regulation of *FLC*, we asked whether FY also transcriptionally regulates *FLC*. As a proxy for transcriptional activity, we used an RT-qPCR assay to measure levels of unspliced *FLC* nascent transcript (ref. 10, Fig. 1*A*). Both unspliced and spliced levels of *FLC* accumulated to higher levels in the hypomorphic *fy-2* allele compared with the parental Columbia line, similar to what was shown for

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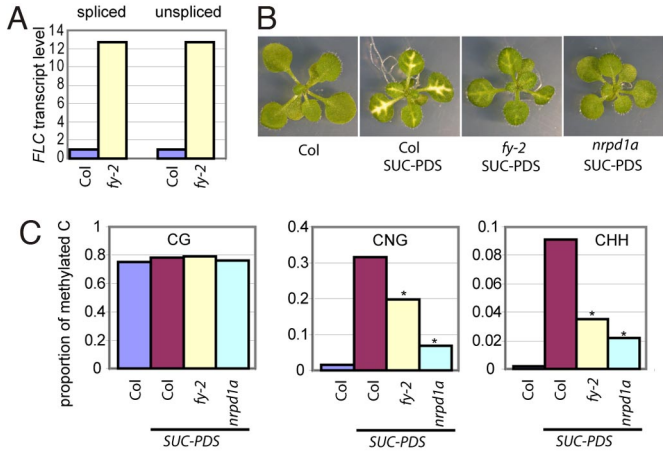
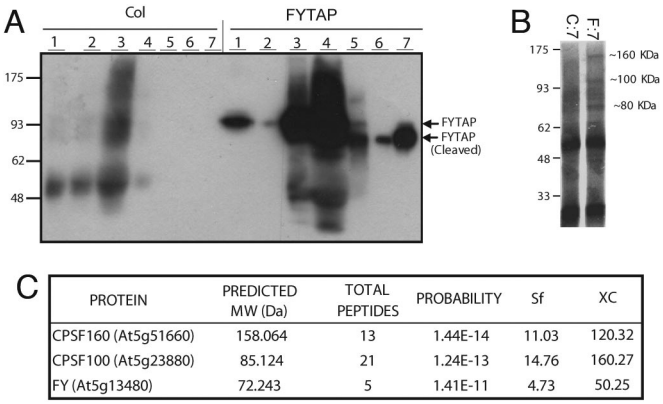


Fig. 1. Characterization of transcriptional defects in *FLC* repression and *SUC-PDS* induced RNA directed DNA methylation in *fy-2*. (*A*) qRT-PCR analysis of *FLC* transcript species. Expression values are shown normalized to Col. (*B*) Photobleaching phenotype. SUC-PDS lines carry a phytoene desaturase (*PDS*) hairpin specifically expressed in the phloem companion cells. The silencing spreads from the vein into adjacent tissue and silences endogenous *PDS*. This is suppressed by *fy-2* and *nrpd1a*. (*C*) CNG and CHH methylation at the endogenous *PDS* locus. * denotes significant differences to the *SUC2-PDS* Col control at $P < 0.01$ (χ^2 test).



PROTEIN	PREDICTED MW (Da)	TOTAL PEPTIDES	PROBABILITY	Sf	XC
CPSF160 (At5g51660)	158.064	13	1.44E-14	11.03	120.32
CPSF100 (At5g23880)	85.124	21	1.24E-13	14.76	160.27
FY (At5g13480)	72.243	5	1.41E-11	4.73	50.25

Fig. 2. FYTAP purification. (*A*) Western analysis at different steps- 1: Input (0.01% of total soluble protein), 2: Unbound (0.01% of total), 3: IgG-beads(0.1% of total), 4: IgG precipitated after elution with TEV protease (1% of total), 5: Calmodulin-bead bound after EGTA elution (5% of total), 6: Calmodulin-bead immunoprecipitate (1% of total), 7: Final elution (5% of total). (*B*) Silver-stained gel of the immunoprecipitated fractions. Step 7 (as in *A*) of FYTAP and Col were size fractionated on a Tris·HCl 4−15% gel and silver stained. (*C*) Summary table for mass-spectral sequencing analysis of FYTAP-tag purification IP-specific peptides. Probability, represents the statistical likelihood of finding an equally good peptide match by chance. XC is an abbreviation for the raw cross-correlation score of the *Top* candidate peptide or protein for a given input data file. The Sf score for each peptide is calculated by a neural network algorithm that incorporates the Xcorr, DeltaCn, Sp, RSp, peptide mass, charge state, and the number of matched peptides for the search (not all of these scores are presented). The higher the Sf score the better the match, a peptide Sf is always <1.

fca-9 and *fld-4* (10). Thus, we conclude that FY, like FCA and FLD, regulates *FLC* expression cotranscriptionally.

We next analyzed whether FY plays a more general role in chromatin silencing by testing whether *fy-2* mutants suppress silencing mediated by the *SUC-PDS* reporter, which has been shown to require *FCA* and *FLD* (5, 16). In this assay, system, a hairpin complementary to parts of the phytoene desaturase (PDS) coding region is expressed specifically in the phloem and a silencing signal moves from the veins into the adjacent mesophyll tissue, where it silences the endogenous PDS causing photobleaching. We crossed the *SUC-PDS* transgene into the *fy-2* mutant background and found that *fy-2* partially suppresses the silencing of endogenous *PDS* to a similar extent as *fca-9* (ref. 5, Fig. 1*B*). As in *fca-9* mutants, we did not find an effect on the abundance of transgene derived sRNA in *fy-2*, suggesting that FY acts downstream of sRNA generation, possibly at the perception and/or interpretation of the silencing signal. To test this hypothesis, we analyzed the DNA methylation at the endogenous PDS in dissected leaf material enriched for silenced tissue using bisulfite sequencing.

SUC-PDS-mediated silencing is strongly suppressed and corresponding DNA methylation strongly decreased in mutants required for RNA-directed DNA methylation (RdDM) such as the largest subunit of the plant-specific DNA polymerase IVa, *nrpd1a*. Thus, *nrpd1a* mutants were included as a control for loss of DNA methylation (Fig. 1*C*). As reported, symmetric CG methylation was unaffected by the *SUC-PDS* transgene, whereas asymmetric DNA methylation in both CNG and CHH sequence contexts was strongly induced by the presence of the transgene (Fig. 1*C*, ref. 5). In *fy-2* mutants, CNG and CHH methylation were both strongly reduced (CNG: 31.6% in *SUC-PDS* compared with 19.9% in *fy-2 SUC-PDS*; CHH: 9.1% in *SUC-PDS* compared with 3.5% in *fy-2 SUC-PDS*; Fig. 1, Table S1). Consistent with weaker suppression of photobleaching in *fy-2* compared with *nrpd1a*, asymmetric DNA methylation in *fy-2* was decreased slightly less than in *nrpd1a*. The loss of DNA methylation in *fy*-2 is similar to what was described for *fca* (5), suggesting that FY acts closely with FCA also in this system. *fy*-2 had no effects on transgene expression or on endogenous PDS expression in the absence of the hairpin transgene. It would, therefore, appear that *PDS* becomes a target for FY only after association of the endogenous gene/transcript with a silencing signal that has spread from an adjacent cell.

FY Forms a Stable Complex with AtCPSF100 and AtCPSF160 in Vivo, But Not with FCA. To identify additional components working together with FY, we chose to undertake a tandem affinity purification (TAP) strategy (17) and fused the CTAPi tag (18) to the C terminus of an FY cDNA. This was then cloned between the FY promoter and 3′ region to generate a construct aimed at expressing FY at wild-type levels and expression pattern. This construct rescued the *fy-2* mutant phenotype with regard to flowering time and *FLC* levels (Fig. S1). The individual steps of the FYTAP complex purification were monitored and optimized by Western blot analysis using FY-specific antibodies until a strong FY-positive signal was obtained in the final eluate (Fig. 2*A*). Extracts from these plants taken through the TAP protocol reproducibly showed enrichment of proteins ≈160, 100, and 80 kDa not detected in WT Col samples (Fig. 2*B*). The final eluate was then taken through LC-MS analysis that revealed multiple peptides not present in control samples corresponding to core RNA processing factors AtCPSF100 (At5g23880) and AtCPSF160 (At5g51660) (Fig. 2*C*, Table S2). FY, the tagged protein, was not represented by most tryptic fragments suggesting tight association of AtCPSF160 and CPSF100 with FY.

In yeast, the *Arabidopsis* FY homologue Pfs2p interacts with Ysh1p (homologous to the mammalian protein CPSF73), Fip1p and a protein of the CstF complex, RNA14p (homologous to mammalian CstF77) (13). However, no peptides from these proteins were detected in our FYTAP complex purification. Our in vitro interaction analyses between AtCstF77 or AtCPSF73 and FY, FY yeast 2-hybrid screening, and an extensive pairwise yeast 2-hybrid interaction analysis between *Arabidopsis* 3′ RNA processing factors (19) also failed to find the expected pairwise interactions between FY and other RNA processing compo-

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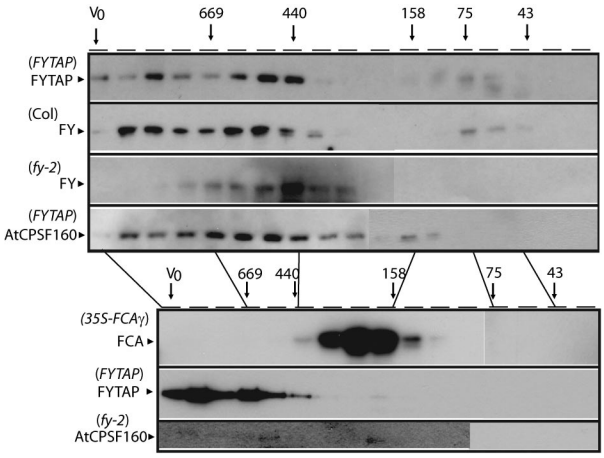

Fig. 3. Western blot analysis of size exclusion chromatography fractions. Protein extracts of indicated genotypes (in parentheses, left) were loaded onto Superdex M200 columns (Amersham Pharmacia) and presence of indicated proteins (arrowhead, left) were assayed by Western blotting. Elution profile of void volume (V_0) and size standards used for calibration is indicated above (values in kDa). Two different Superdex M200 columns were used so the Western image is centered on the 440-kDa size standard.

nents. However, FY has been found copurifying with AtCPSF100, CLPS3 (11, 20) and other RNA processing factors in apparently distinct complexes.

The FCA/FY interaction is well characterized in vitro and can be reproduced by using FCA/FY counterparts from other plant species (9, 21). Furthermore, this interaction was instrumental to the cloning of *FY* (8). We were therefore expecting to find FCA peptides in the FYTAP purification; however, none were detected. In addition, attempts to verify the FCA/FY interaction in vivo by direct coimmunoprecipitation using either FCA or FY as baits and reciprocal antibodies failed even when stringency criteria were reduced compared with the FYTAP purification. This could reflect different stringencies of methods to detect interactions or biological complexity not recapitulated in vitro.

Distribution of FY Protein Complexes Changes in the Hypomorphic Mutant *fy-2*. To provide further insight into in vivo protein interactions of FY, we used size exclusion chromatography to analyze the protein complexes containing RNA processing components. Western analysis of different fractions using the anti-TAP antibody revealed multiple FY complexes clearly differing in size (Fig. 3). The larger complexes were present in fractions corresponding to ≈800 kDa and the smaller ones in fractions corresponding to ≈500 kDa. This pattern of FY complexes was also found in nontransgenic Columbia extracts analyzed using the FY polyclonal antibody (Fig. 3), indicating it was not an artifact of using the tagged protein. Assaying the same blots with an anti-AtCPSF160 antibody (22) revealed AtCPSF160 was present in a large range of fractions including those containing FY, supporting the notion that the multiple FY-containing complexes also contain other AtCPSF machinery (Fig. 3). An anti-AtCPSF100 antibody (22) was used, but no signal was detected on the Western blots, so AtCPSF100 presence could not be assayed.

We next asked whether FCA could also be present in fractions carrying FY/AtCPSF160 complexes. The specific FCA antibody revealed FCA protein in fractions migrating at ≈200kDa, both in lines expressing a 35S-FCAγ transgene (23) (Fig. 3) and wild-type plants. There did not appear to be any overlap between FY/AtCPSF160 containing fractions and those containing FCA. This is consistent with the results of our FYTAP purification where FCA was not detected and furthermore lends support to the hypothesis that the FCA/FY interaction in vivo may be regulated or transient.

Analysis of null *fy* alleles showed that *FY*, like *Pfs2*, is an essential gene (9). This is also the case for both FY interactors AtCPSF160 and AtCPSF100 (24). However, all currently available *fca* alleles and hypomorphic *fy* alleles expressing the conserved N-terminal 7 WD repeats but lacking the FCA interaction domain are viable but late flowering (9). In light of the identification of multiple FY/AtCPSF containing complexes, we considered that FY might undertake multiple functions in vivo, some perhaps as part of a "housekeeping" complex performing essential functions and others as part of specialized FCA-associated functions. To test this idea, we exploited the hypomorphic *fy*-2 allele that expresses a truncated FY unable to interact with FCA. Strikingly, the ratio of the different complexes was substantially changed, with the smaller FY complexes being much more predominant in the *fy-2* mutant (Fig. 3). It is possible, therefore, that a subset of the complexes is involved in more specialized functions, such as FCA-mediated chromatin silencing, although FCA is not an integral part of those complexes.

***fy* Mutations Localized to the FCA Interaction Domain Influence FY/AtCPSF160 Complexes.** To pursue the biological role of the FCA/FY interaction, we sought to identify novel *fy* alleles that specifically affected the FCA-binding motif in FY (9). To this end, we exploited a mutagenesis strategy used to identify components required for FCA-mediated *FLC* down-regulation. Suppressors of overexpressed FCAγ (*sof*) mutants were identified based on increased *FLC* levels in a background expressing a *35S-FCAγ* transgene. (Fig. 4*A*) The identification of the mutants is facilitated by the use of an *FLC-luciferase* (*FLC-LUC*) translational fusion (10). The *sof28* and *sof44* mutants show high FLC-LUC reporter activity (Fig. S2*A*). Consistently, endogenous *FLC* levels (Fig. 4*A*) are increased through transcriptional effects (Fig. S2*B*) and flowering is delayed (Fig. S2*C*). This suggests that the mutations disrupt the regulation of endogenous *FLC*, and that their effect on FCA-mediated *FLC* repression is not specific for the FLC-LUC reporter.

Sequencing of *FY* cDNA revealed mis-sense mutations affecting the first FY PPLP motif (Fig. 4*B*). Both mutations changed a CCT base triplet coding for proline to TCT coding for serine instead. The *sof44* mutation changed P488 in FY to serine and thus PPLP to SPLP, the *sof28* mutation changed P489 to serine and consequently PPLP to PSLP (Fig. 4*B*). We confirmed that the mutations in *FY* are responsible for the phenotype by crossing the previously characterized *fy-1* allele with *sof28* and *sof44* and the progenitor control and tested the phenotype of the F1 generation (Fig. S2*D*). Heteroallelic combinations between *fy-1* and *sof28* or *sof44* showed significantly increased FLC-LUC reporter activity. We conclude that the novel mutations in *FY* are causing the phenotype, and that *sof28* and *sof44* are new *fy*-alleles. Compared with the *fy-1* mutant that expresses a protein lacking most of the variable C-terminal domain (Fig. 4*B*), the phenotypic consequences of the amino acid substitutions found in the PPLP-disrupted alleles might be expected to be comparably weak. However, our analysis of endogenous *FLC* (Fig. 4*A*) and flowering time analysis of heteroallelic combinations between the PPLP mutants and *fy-1* that did not lead to a further delay in flowering suggest they are similarly strong (Fig. S2 *C* and *E*). We therefore tested whether the PPLP mutations generally destabilized the FY protein but found that FY levels are not reduced compared with controls (Fig. S2*F*). Taken together, we conclude that mutations that specifically disrupt the function of the first FY PPLP motif have similar phenotypic strength to *fy-1*, and that the first PPLP motif is necessary for FCA-mediated *FLC* suppression in vivo.

The functional requirement for the PPLP motif prompted us

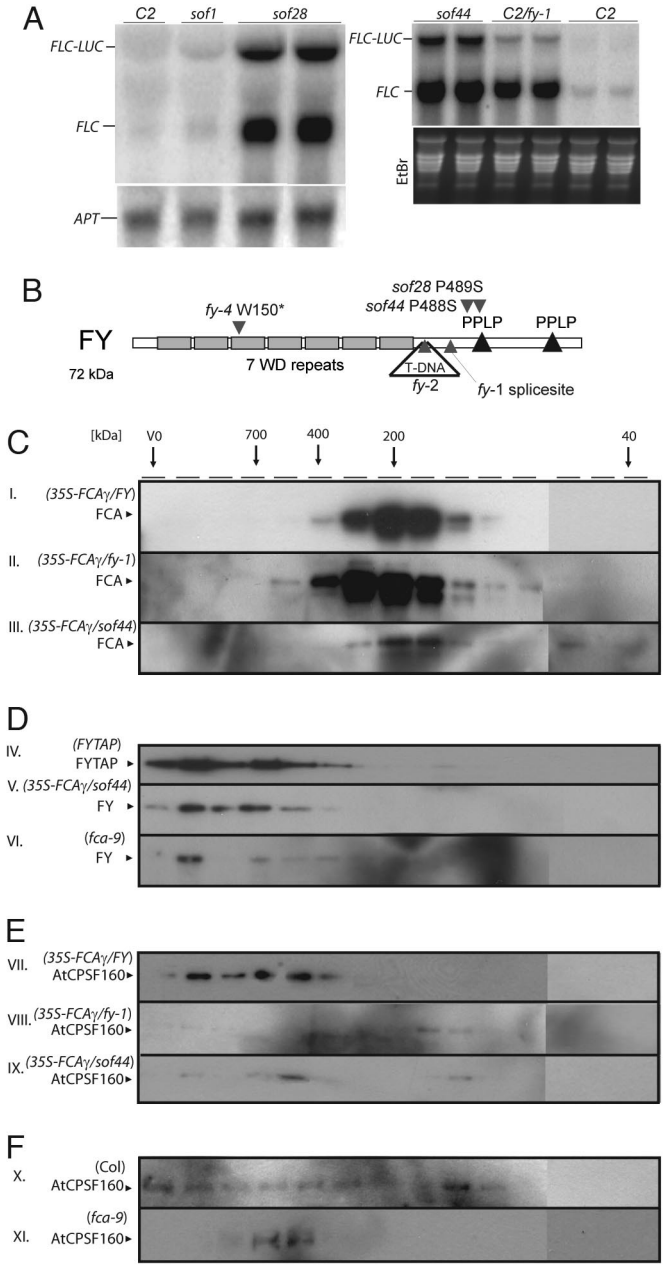

Fig. 4. Identification of *FY* PPLP alleles (*sof28* and *sof44*) and their effect on FY/AtCPSF complex formation. (*A*) Northern blot analysis of *FLC* and *FLC-LUC* transcripts. C2 is the progenitor line used for mutagenesis - *35S-FCAγ*, *FRI (JU223)* and *FLC-LUC*. *sof1* is a *fld* allele (10). (*B*) Schematic representation of FY. Seven N-terminal WD-repeats (boxes) and 2 C-terminal PPLP motifs (black triangle) are shown. Gray triangles represent characterized mutations in FY, nonsense mutation found in *fy-4* indicated by (*). (*C–F*) Soluble protein extracts were loaded onto Superdex M200 size-exclusion columns and the size distribution of protein complexes analyzed by Western blot analysis. (*C*) Size distribution of FCA containing protein complexes (I–III). (*D*) Size distribution of FY containing protein complexes (IV–VI). (*E*) Size distribution of AtCPSF160 containing protein complexes in C2 background (VII–IX). (*F*) Size distribution of AtCPSF160 containing protein complexes without 35S-FCAγ transgene (X–XI).

to analyze the importance of the FCA/FY interaction in the context of FY/AtCPSF and FCA complex formation (Fig. 4). The elution profile of the FCA complex was unchanged in PPLP mutants or in *fy-1*(Fig. 4*C* I–III). The elution profile of FY complexes was unchanged in PPLP mutants or *fca-9* (Fig. 4*D* IV–VI); however, the profile of the AtCPSF complexes was significantly changed. The abundance of the higher MW complexes was reduced in *fy-1* and a PPLP mutant (Fig. 4*E* VIII and IX compared with VII), and this was also observed in *fca-9* (Fig. 4*F* XI compared with X); both results strengthen the argument that reduction in the abundance of the high MW AtCPSF complex in *fy-2* (Fig. 3) may be attributed specifically to the loss of the FCA/FY interaction. It is also possible that overexpression of FCA through the *35S-FCAγ* transgene (Fig. 4*E* VII compared with Fig. 4*F* X and Fig. 3) promotes formation of higher MW AtCPSF complexes. The different effect of these genotypes on the FY and AtCPSF160 profiles reinforces the view that multiple RNA processing complexes of differing composition occur in vivo. Taken together, our data suggest that the FCA/FY interaction leads to altered interactions in the FY/AtCPSF complexes and this is a necessary step in FCA-mediated chromatin silencing.

Discussion

Our interest in FY, the *Arabidopsis* homologue of the yeast polyadenylation/RNA 3′ processing factor Pfs2p, was precipitated through its role in flowering time control. We cloned FY through its interaction with FCA, an RNA-binding protein, and showed this interaction led to down-regulation of the floral repressor gene *FLC* (8). Consistent with a posttranscriptional role, FY was found to be required for alternative polyadenylation of the *FCA* transcript (25). We therefore considered that FCA might be specifically recruiting transcripts to the cellular RNA 3′ processing machinery that includes FY, to effect a posttranscriptional regulation. However, suppressor mutagenesis and chromatin analysis later revealed FCA regulates *FLC* through a transcriptional silencing mechanism via the histone demethylase activity of FLD (10). FCA also plays more general roles in silencing transgenes and transposons in the *Arabidopsis* genome (5). To understand the connection between the RNA processing activities and chromatin regulation, we have pursued FY roles in *Arabidopsis*. Here, we show that, in common with *fca* and *fld* alleles, mutations in this conserved RNA processing homologue cause defects in transcriptional regulation of *FLC* and transgene-mediated DNA methylation. We also describe tandem affinity purification to identify AtCPSF100 and AtCPSF160 as stably associated FY in vivo interactors. We show that in wild-type plants, FY/AtCPSF160 form multiple protein complexes, with the abundance of the larger complexes being significantly reduced in extracts from the *fy-2* mutant, in which FY is present as a truncated protein able to complement the essential FY functions but unable to interact with FCA. Surprisingly, given the FY/FCA physical interaction, FCA did not appear to be part of either FY complex but was detected in a complex of ≈200 kDa. Loss of FCA function or loss of the FCA interaction domain in FY disrupted formation of the larger complexes containing AtCPSF160 but not those containing FY. Overexpression of FCA also appeared to promote formation of higher MW AtCPSF160 complexes. These data suggest that the *Arabidopsis* 3′ RNA processing components FY/AtCPSF160 exist in functionally distinct complexes in vivo and that one step in FCA-mediated chromatin silencing is a transient or regulated FCA-FY interaction leading to changes in FY/AtCPSF complex composition.

A role for RNA in chromatin regulation has received much attention in the last few years. However, little information is available that mechanistically links RNA processing components and chromatin regulation. In *Saccharomyces cerevisiae*, the RNA 3′ end processing factor Swd2p has been found to function in both 3′ end cleavage and global histone modification (26), although these functions seemed independent. Recent reports have also provided evidence for physical interaction between the Paf1 complex, implicated in transcriptional elongation control, and CPSF/CstF components, perhaps facilitating access of the

3′end processing machinery to actively transcribed regions (27, 28). Our work on the *Arabidopsis* floral repressor gene *FLC* leads us to speculate that FY, a conserved RNA processing factor, encoded by a single copy *Arabidopsis* gene, functions both in housekeeping posttranscriptional processes and in chromatin regulation. In this scenario, the essential functions of FY, together with AtCPSF160 and AtCPSF100, would require the FY/AtCPSF complexes of ≈500 kDa. Formation of the ≈800 kDa complexes, stimulated through cotranscriptional interaction of FY with FCA (the RNA-binding protein), would be involved in chromatin regulation. These would function at *FLC* in an FLD (the histone demethylase)-dependent transcriptional regulation (10). This function is not limited to *FLC*, as *fy-2* mutants are not only late-flowering but also defective in the DNA methylation linked to silencing of an endogenous gene in response to systemic silencing signal emanating from the vasculature.

Defects in RNA processing components have been associated with an enhanced silencing phenotype (11). By expressing viral sequences from an integrated transgene, *esp* mutants were identified as being defective in the cellular recognition of the viral sequences. This led to increased viral replication and higher levels of PDS small RNAs, which in turn enhanced the silencing of the endogenous PDS gene (11). If FY, as proposed for FCA (5), is in part required to recognize the silencing signal at the endogenous PDS locus and trigger silencing, then the ESP loci and FY are actually having functionally similar primary effects in both assay systems, despite apparently opposite effects of *esp* and *fy* mutations on flowering time. Other *Arabidopsis* cotranscriptional processes may also participate in chromatin silencing given the identification of the splicing factor *PRP2* as an *ESP* locus (11) and the recent finding that splicing factors facilitate RNAi-directed silencing in fission yeast (12). A key next question will be to determine what aspect of the RNA processing function of FY is involved in directing the chromatin silencing machinery to the specific locus.

The FY/FCA interaction, which is clearly needed in *Arabidopsis* for correct *FLC* repression and *FCA* autoregulation, may be regulated in vivo, so it is interesting that some WW domain interactions have been shown to require phosphorylation of the proline-rich ligands (29). The FCA–FY interaction appears to be conserved generally in plants, because it has been demonstrated in rice and *Arabidopsis* (21). Evolution of the FCA–FY interaction, perhaps through addition of the C-terminal region to an ancestral FY-like protein, would, through a modularity of protein complexes, lead to new functions for RNA processing components. These new functions may not be limited to plants, because FY homologues from all organisms, apart from *S. cerevisiae* and *Schizosaccharomyces pombe*, have significant C-terminal extensions (9). These C-terminal domains are unrelated in the different organisms and vary greatly in length. The vertebrate homologues (HsWDC146, TnWDC146) display especially extended domains, consisting of low complexity sequence rich in proline, glycine, and glutamine residues (30), and the testes-specific expression pattern of HsWDC146 (the closest FY homologue) suggests a developmental function for this protein (30). The *Neurospora* FY homologue C-terminal domain contains 2 PPLP motifs, suggesting there may also be conservation in the type of protein interactions.

Future work may address the full molecular composition of the larger FY/AtCPSF160 processing complexes and how it changes upon the FCA-FY interaction. We also need to determine how that activity recruits the chromatin silencing functions. It will be important to see how this cotranscriptional mechanism integrates with other pathways that carry out processing and quality control of transcripts before they are exported from the nucleus.

Materials and Methods

Constructs and expression analysis A 400-bp PCR-amplified FY probe was made by using primers FY1 5′-CAATGTACGCCGGCGGCGATA-3′and FY4 5′-ACGGTTGATTGAACAACGGTTC-3′. An *FLC* probe was made by using primers FLC 3′UTR3′: 5′-ACGAATAAGGTACAAAGTTC-3′ and *FLC* exon7 5′-GGAGAATAATCATCATGTGGGAGCA-3′, and Northern blot analysis was performed as described (10). Spliced/unspliced *FLC* transcripts were determined as described (5). FY Western blot analyses were performed as described in ref. 9. The TAP epitope was detected by using PAP antibodies (Sigma P1291) at 1:1,000 dilution. AtCPSF160 detection was performed by using anti-AtCPSF160 antibodies (22) at 1:1,000 dilution. Generation of the FY-TAP construct is detailed in *SI Methods*.

TAP Tag Purification and Gel Filtration. The TAP purification protocol (17) and gel filtration analyses were used as described in ref. 31. Identification of proteins in the TAP eluates by mass spectrometry are described in the *SI Methods*.

SUC-PDS Silencing and DNA Methylation. *fy-2* was crossed into the SUC-PDS transgenic line (16), and doubly homozygous plants were selected for phenotypic analyses. Bisulfite sequencing of the endogenous PDS gene was performed on dissected primary leaves enriched for silenced tissue as described in ref. 5. Nineteen to thirty-two independent clones were analyzed per genotype (see Table S1).

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1. Moazed D (2009) Small RNAs in transcriptional gene silencing and genome defence. *Nature* 457:413–420.
2. Michaels SD, Amasino RM (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* 11:949–956.
3. Michaels SD, Amasino RM (2001) Loss of FLOWERING LOCUS C activity eliminates the late- flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* 13:935–941.
4. Sheldon CC, et al. (1999) The FLF MADS box gene: A repressor of flowering in Arabidopsis regulated by vernalization and methylation. *Plant Cell* 11:445–458.
5. Bäurle I, Smith L, Baulcombe DC, Dean C (2007) Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. *Science* 318:109–112.
6. Bäurle I, Dean C (2006) The timing of developmental transitions in plants. *Cell* 125:655–664.
7. Bäurle I, Dean C (2008) Differential interactions of the autonomous pathway RRM proteins and chromatin regulators in the silencing of *Arabidopsis* targets. *PLoS ONE* 3:e2733.
8. Simpson GG, Dijkwel PP, Quesada V, Henderson I, Dean C (2003) FY is an RNA 3′ end-processing factor that interacts with FCA to control the Arabidopsis floral transition. *Cell* 113:777–787.
9. Henderson IR, Liu F, Drea S, Simpson GG, Dean C (2005) An allelic series reveals essential roles for FY in plant development in addition to flowering-time control. *Development (Cambridge, UK)* 132:3597–3607.
10. Liu F, et al. (2007) The *Arabidopsis* RNA-binding protein FCA requires a lysine-specific demethylase 1 homolog to downregulate FLC. *Mol Cell* 28:398–407.
11. Herr AJ, Molnar A, Jones A, Baulcombe DC (2006) Defective RNA processing enhances RNA silencing and influences flowering of *Arabidopsis. Proc Natl Acad Sci USA* 103:14994–15001.
12. Bayne EH, et al. (2008) Splicing factors facilitate RNAi-directed silencing in fission yeast. *Science* 322:602–606.
13. Ohnacker M, Barabino SM, Preker PJ, Keller W (2000) The WD-repeat protein pfs2p bridges two essential factors within the yeast pre-mRNA 3′-end-processing complex. *EMBO J* 19:37–47.
14. Zhao J, Hyman L, Moore C (1999) Formation of mRNA 3′ ends in eukaryotes: Mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Microbiol Mol Biol Rev* 63:405–445.
15. Yao Y, Song L, Katz Y, Galili G (2002) Cloning and characterization of Arabidopsis homologues of the animal CstF complex that regulates 3′ mRNA cleavage and polyadenylation. *J Exp Bot* 53:2277–2278.
16. Smith LM, (2007) An SNF2 protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in *Arabidopsis. Plant Cell* 19:1507–1521.
17. Rigaut G, et al. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* 17:1030–1032.
18. Rohila JS, Chen M, Cerny R, Fromm ME (2004) Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. *Plant J* 38:172–181.

19. Hunt AG, et al. (2008) *Arabidopsis* mRNA polyadenylation machinery: Comprehensive analysis of protein–protein interactions and gene expression profiling. *BMC Genomics* 9:220.
20. Xing D, Zhao H, Li QQ (2008) *Arabidopsis* CLP1-SIMILAR PROTEIN3, an ortholog of human polyadenylation factor CLP1, functions in gametophyte, embryo, and postembryonic development. *Plant Physiol* 148:2059–2069.
21. Jang YH, Lee JH, Kim JK (2008) Abscisic acid does not disrupt either the *Arabidopsis* FCA-FY interaction or its rice counterpart in vitro. *Plant Cell Physiol* 49:1898–1901.
22. Xu R, et al. (2006) The 73 kD subunit of the cleavage and polyadenylation specificity factor (CPSF) complex affects reproductive development in *Arabidopsis Plant Mol Biol* 61:799–815.
23. Macknight R, et al. (1997) FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. *Cell* 89:737–745.
24. Tzafrir I, ET AL. (2004) Identification of genes required for embryo development in *Arabidopsis. Plant Physiol* 135:1206–1220.
25. Quesada V, Macknight R, Dean C, Simpson GG (2003) Autoregulation of *FCA* pre-mRNA processing controls *Arabidopsis* flowering time. *EMBO J* 22:3142–3152.
26. Dichtl B, Aasland R, Keller W (2004) Functions for S. cerevisiae Swd2p in 3′ end formation of specific mRNAs and snoRNAs and global histone 3 lysine 4 methylation. RNA 10:965–977.
27. Rozenblatt-Rosen O, et al. (2009) The tumor suppressor Cdc73 functionally associates with CPSF and CstF 3′ mRNA processing factors. *Proc Natl Acad Sci USA* 106:755–760.
28. Nordick K, Hoffman MG, Betz JL, Jaehning JA (2008) Direct interactions between the Paf1 complex and a cleavage and polyadenylation factor are revealed by dissociation of Paf1 from RNA polymerase II. *Eukaryot Cell* 7:1158–1167.
29. Sudol M, Hunter T (2000) New wrinkles for an old domain. *Cell* 103:1001–1004.
30. Ito S, et al. (2001) A novel WD40 repeat protein, WDC146, highly expressed during spermatogenesis in a stage-specific manner. *Biochem Biophys Res Commun* 280:656–663.
31. De Lucia F, Crevillen P, Jones AM, Greb T, Dean C (2008) A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of FLC during vernalization. *Proc Natl Acad Sci USA* 105:16831–16836.