Differential Interactions of the Autonomous Pathway RRM Proteins and Chromatin Regulators in the Silencing of Arabidopsis Targets

Isabel Bäurle, Caroline Dean

Department of Cell and Developmental Biology, John Innes Centre, Norwich, United Kingdom

Abstract

We have recently shown that two proteins containing RRM-type RNA-binding domains, FCA and FPA, originally identified through their role in flowering time control in Arabidopsis, silence transposons and other repeated sequences in the Arabidopsis genome. In flowering control, FCA and FPA function in the autonomous pathway with conserved chromatin regulators, the histone demethylase FLD and the MSI1-homologue FVE, a conserved WD-repeat protein found in many chromatin complexes. Here, we investigate how the RRM proteins interact genetically with these chromatin regulators at a range of loci in the Arabidopsis genome. We also investigate their interaction with the DNA methylation pathway. In several cases the RRM protein activity at least partially required a chromatin regulator to effect silencing. However, the interactions of the autonomous pathway components differed at each target analysed, most likely determined by certain properties of the target loci and/or other silencing pathways. We speculate that the RNA-binding proteins FCA and FPA function as part of a transcriptome surveillance mechanism linking RNA recognition with chromatin silencing mechanisms.

Introduction

A significant fraction of eukaryotic genomes comprises repeated sequences including transposons and retroelements. These sequences are effectively silenced through a number of transcriptional and posttranscriptional pathways involving DNA methylation, small RNAs (sRNA) and histone modifications [1,2,3]. Plant DNA is methylated at cytosine bases in the CG, CNG (N is any nucleotide) and CHH (H is A, C or T) contexts [4,5]. CG methylation is efficiently copied onto the daughter strand after DNA replication whereas non-CG methylation requires an active mechanism to re-establish the methylation following replication. For some loci this involves sRNA and the plant-specific RNA polymerase IV (PolIV) [1,6]. Efficient silencing therefore paradoxically involves transcription of the locus [7]. We have recently identified an additional Arabidopsis pathway involved in silencing of several endogenous transposons and retroelements through the finding that the RRM-domain proteins FCA and FPA play a role in RNA-mediated silencing of a transgenic hairpin [8]. Although this pathway is distinct from the sRNA-directed DNA methylation pathway, both pathways interact closely in a target-specific manner [8]. This is particularly evident from the analysis of the transgene system that originally identified the additional function for FCA and FPA [8]. There, FCA and FPA are required for sRNA amplification [8].

FCA and FPA were originally identified based on their role in flowering time control [9,10,11]. Both proteins promote flowering by down-regulating the expression of the gene encoding the MADS-domain protein FLOWERING LOCUS C (FLC), which is the major repressor of flowering in Arabidopsis [12,13]. FCA and FPA both contain multiple RRM-domains but share no other sequence homology. Flowering is closely aligned with seasonal conditions and most pathways impacting on flowering rely on environmental cues such as temperature and photoperiod (reviewed in [14]). fca and fpa mutants still respond well to environmental cues and were for this reason put into a group named the autonomous pathway (AP). This group also comprises two chromatin regulators, the putative histone H3 K4 histone demethylase FLOWERING LOCUS D (FLD), which is a homologue of human LSD1, and the MSI1 homologue FVE [15,16,17]. FVE is one of five Arabidopsis MSI1-like genes, which are homologous to the eukaryotic MSI1 family of WD40 domain-containing proteins found in several protein complexes acting on chromatin [18]. The autonomous pathway also comprises the homeodomain protein LUMINIDEPENDENS (LD) [19], the K homology-domain protein FLOWERING LATE WITH KH MOTIFS (FLK) [20,21] - also a putative RNA-binding protein - and FY, a homologue of the S. cerevisiae 3'-end processing/ polyadenylation factor Pfs2p [22].

The interactions of the AP components FCA, FY and FLD have been analysed [22,23]. FCA negatively regulates its own expression through alternative transcript 3’ processing, and this and its regulation of FLC requires a physical interaction with FY [22,24]. FCA also regulates the activity of the histone demethylase FLD to down-regulate FLC, suggesting an RNA metabolism/ processing step triggers chromatin changes at FLC [23].

Here, we have continued to investigate the role of the AP in chromatin silencing, and have focused on the functional
interactions of the RRM-domain proteins FCA and FPA with the chromatin regulators FLD and FVE. We show that FVE, FLD and the third putative RNA-binding protein, FLK, also play a widespread role in chromatin silencing and that they interact functionally in a target specific manner. We also show that the RRM protein FPA largely acts through the histone demethylase FLD in the silencing of FLC, reinforcing the conclusion that RRM-type RNA-binding proteins trigger a chromatin change to effect silencing. We find that the interactions of the RRM proteins and the chromatin regulators are different at each target and we exemplify this by comparing FLC and AtMu1 regulation.

### Results and Discussion

The RRM-domain protein FPA acts through the histone demethylase FLD to suppress FLC expression

We had previously shown that the RRM protein FCA requires both the 3' processing/polyadenylation factor FY [22] and the histone demethylase FLD to down-regulate FLC [23]. To address whether the second RRM protein, FPA, also requires other AP components for its function, we generated plants expressing FPA from a genomic fragment under the control of the constitutive 35S promoter. The 35S::FPA construct complemented an fpa mutant background.

**Figure 1.** Overexpression of FPA in autonomous pathway mutant backgrounds and FRI. For each background a non-transformed (nt) control and T2 generation plants from 1–3 independent transformed lines (numbers) were used to assay flowering time and expression levels of FLC, FPA and APT by RNA gel blot analysis. fpa-8 and FRI did not flower during the experiment, which was terminated at ~70 leaves. Error bars indicate standard error of the mean. Lines were processed in two separate experiments as indicated. Within one experiment, all RNA gel blot panels shown come from the same membrane/ hybridization.

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**Figure 2.** Flowering time of autonomous pathway single and double mutants. Flowering time of the indicated mutant plants grown under long day conditions in a greenhouse was measured in days to flowering (opening of the first flower). Flowering time is indicated as the average delay in flowering relative to the Col wild type +/- standard error of the mean. (yellow (fve), green (fld), blue (flk), and black (all others) bars). Col was flowering at 39 days. White and grey bars indicate predicted flowering time for calculated additive and epistatic scenarios, respectively. *, additive interaction; **, epistatic interaction.

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with respect to FLC transcript levels and flowering time and was thus considered fully functional (Figure 1). Overexpression of FCA suppresses late flowering and high FLC expression levels caused by the presence of the strong FLC activator FRI [23]. Similarly, we found that overexpression of FPA in a FRI background repressed FLC expression levels and resulted in early flowering, thus confirming that FPA overexpression is sufficient to overcome even high FLC levels (Figure 1). We then studied whether any of the AP components FCA, FLD, FLK, FVE, or FV were required for the FPA-mediated repression of FLC. FPA overexpression reduced flowering time and FLC levels in fca, flk, fca and fy mutant backgrounds to the wild type level, suggesting that these genes are not required for FPA function on FLC. However, overexpression of FPA in an fld mutant background reduced both flowering time and FLC levels only slightly compared to non-transformed fld mutant plants, suggesting that FPA acts in part through FLD. This is further supported by the finding that fpa/fld double mutants flowered at the same time as the later of the single mutants (Figure 2) and our previous results demonstrating that reactivation of FLC in fpa and fld mutants is at the level of transcription [8,23]. Together, the finding that the RRM-domain protein FPA represses FLC expression through the putative histone demethylase FLD is in line with a model where an RNA-binding component recognizes a particular RNA feature and this triggers chromatin silencing of the locus. Interestingly, while FCA requires both FY and FLD, FPA requires FLD but not FY to repress FLC, indicating that the involvement of the histone demethylase FLD is common to both RRM-proteins, while the interaction with the 3′-end processing factor FY is specific to FCA.

Figure 3. Expression levels of (retro)transposon targets in AP single and double mutants. Expression levels were determined by quantitative RT-PCR for AtMu1 (A) and IG/LINE (B). Data from 3 independent biological replicates were averaged and normalized to Col, ±/− standard error of the mean. (C) AtSN1 expression levels were determined by semi-quantitative RT-PCR (AtSN1, 36 cycles; TUB, 25 cycles), a representative experiment from 3 independent replicates is shown. doi:10.1371/journal.pone.0002733.g003
Analysis of flowering time in double mutants

To reveal further interactions between components of the autonomous pathway, we created a number of double mutant combinations in the Columbia (Col) background. We chose Col over the Landsberg erecta accession, which had been used for the early genetic work on the autonomous pathway [11,25], because Landsberg erecta FLC carries a transposon insertion in the 3' end of its first intron and this results in a reduction of expression of the locus through sRNA-mediated silencing [26]. We determined flowering time of the single and double mutants under long days (Figure 2). Previous studies have established that the delay in flowering in AP mutants is caused by the misregulation of FLC [13,15,27]. All double mutants flowered at least as late as the later single mutant and many considerably later, as expected if independent action of both mutants was assumed. To further analyze the interaction of the studied mutants during flowering time control, we calculated predicted flowering times for the two simplest genetic interactions conceivable and compared them to the experimentally obtained values. If both gene actions were fully independent, we would expect additivity of the delay in flowering in the double mutant (\(\Delta m_1 m_2 = \Delta m_1 + \Delta m_2\)). If one gene action was dependent upon another (epistasis), we would expect the double mutant to flower at the same time as one parent (eg. \(\Delta m_1 m_2 = \Delta m_1\), for \(\Delta m_1 > \Delta m_2\)). This method indicated that fca flk have an epistatic interaction and fca fve are additive, confirming previous findings [23]. It also indicated fve fpa are epistatic and both fca flk and fpa flk additive, thus complementing the overexpression experiments described above. The flowering time of the remaining double mutants, including fpa fve, was intermediate between the two scenarios, suggesting more complex interactions. Thus, in flowering time control, FCA and FPA both act (at least partly) through the histone demethylase FLD, while FVE acts independently of FCA, but may have a more complex interaction with FPA. Finally, the putative RNA-binding protein FLK acts independently of both FCA and FPA.

Derepression of AtMu1, AtSN1 and IG/LINE in double mutants

We have recently found that FCA and FPA also regulate a range of other loci that are subject to sRNA-dependent chromatin silencing [8]. We therefore investigated the interactions of the AP components in their regulation. We first analyzed transcript levels of AtMu1, AtSN1 and IG/LINE [6,28,29] in fca, fpa, fve, flk and fpa flk double mutants as well as Col FRI plants (Figure 3). While AtMu1 showed only a slight reactivation of expression in most AP mutants, it was very highly up-regulated in fve (16.5 fold over wild type; Figure 3A and [8]). IG/LINE expression was only enhanced in fve (Figure 3B), whereas AtSN1 expression was strongly increased in fpa, and slightly increased in fve and flk mutants (Figure 3C). Our previous analysis indicated redundancy between FCA and FPA in the regulation of these additional targets [9]. However, it was not clear whether this reflected their shared feature of RRM-domains in particular or whether double mutants with other AP components would also show more-than-additive effects. We therefore analyzed the available double mutants in the Col background, focussing on the RRM-domain proteins FCA and FPA and the chromatin regulators FVE and FLD. Indeed, a number of these double mutants showed stronger reactivation of AtMu1, AtSN1 and IG/LINE than any of the single mutants. Most noticeably, fpa fve showed a 4.5-fold increase in expression of the DNA transposon AtMu1 over fve (73-fold over wild type, Figure 3A). fca fve and fca flk showed an increase in AtMu1 expression compared to wild type but less than fve alone. The significance of this reduction in these double mutants is at present unclear. fpa flk and fpa flk both showed slightly higher AtMu1 expression than any of the respective single mutants. Unexpectedly, fpa flk mutants (but not fpa fve or fve fpa mutants) consistently showed hyper-repression of AtMu1 expression (5-10-fold).

IG/LINE is an intergenic transcript flanked by a solo LTR which presumably acts as a promoter element [29]. Despite lack of IG/LINE reactivation in any of the single mutants with the exception of a slight increase in flk, in the majority of double mutants tested IG/LINE expression was reactivated (Figure 3B). This indicates a function in IG/LINE repression for all the AP mutants tested here, most obviously seen in fpa flk, fca fve and fve fve.

The retroelement AtSN1 is reactivated in all double mutants with fpa, fve or flk to an extent which approximately reflects the addition of the reactivation in the respective single mutants (Figure 3C). The one exception was fpa flk which displayed a strong synergistic reactivation of AtSN1. Thus, the most obvious conclusion is that FPA, FVE, FLD and FLK act largely independently on AtSN1.

These data therefore reveal the inherent redundancy of AP components. Effects on the targets are in most cases only revealed in double mutant backgrounds and the variation at the different loci presumably reflects their differential interaction with each other and with other silencing pathways.

Figure 4. Interaction of FCA, FPA and FVE on FLC and AtMu1. Overexpression (ox) of FCA or FPA compensated loss of FVE on FLC, but not AtMu1. FLC and AtMu1 expression in the indicated genotypes were determined by quantitative RT-PCR. Error bars indicate standard error of the mean. doi:10.1371/journal.pone.0002733.g004
Differential interactions of FCA, FPA and FVE on FLC and AtMu1

To further dissect the differential interactions between AP components, we analyzed the effect of FCA, FPA and FVE on AtMu1 regulation in more detail and compared it to the situation at FLC. At the FLC locus, overexpression of either FCA or FPA can compensate for the loss of FVE protein and reduce FLC expression to or below wild type levels (Figure 4), suggesting that both FCA and FPA act independently of FVE on FLC. The strong

Figure 5. DNA methylation at AtMu1 in double mutants. (A) Schematic representation of the AtMu1 TIR showing the recognition sites of the enzymes used in (B) and (C), and the type of methylation analyzed. DraI was used as a control for complete digestion in all experiments ((C) and data not shown). (B) and (C) Relative DNA methylation assayed by quantitative PCR of restriction enzyme digested DNA. Error bars indicate standard error of the mean. (D) Bisulfite sequencing of AtMu1 TIR; ddm1, in which DNA methylation is strongly decreased [28], was included as a control. doi:10.1371/journal.pone.0002733.g005
reactivation of AtMu1 in foe enabled us to ask whether the same was true for AtMu1. Overexpression of FCA or FPA in an foe mutant did not restore silencing of AtMu1 (Figure 4), suggesting that either FCA and FPA act through FVE on AtMu1, or that FCA and FPA act in parallel to FVE and overactivation of FCA or FPA is not sufficient to counteract loss of FVE. foe foe did not show higher AtMu1 expression than foe (Figure 5A), consistent with the notion that FCA is working through FVE on AtMu1. By contrast, fpa foe did show higher AtMu1 expression than foe, consistent with independent action of both genes. Furthermore, the foe mutant background was more sensitive (than wild type) to loss of fpa with respect to AtMu1 reactivation, highlighting the idea of redundancy between different AP components.

The autonomous pathway mediates silencing through DNA methylation-dependent and -independent effects

Silencing of AtMu1 is associated with both symmetric (CG) and asymmetric (CNG, CHH) DNA methylation [28,30]. Derepression of AtMu1 in foe fpa correlated with a loss in asymmetric DNA methylation [8] and Figure 5B). To address whether a similar loss in DNA methylation at AtMu1 occurred in other AP mutants with AtMu1 mis-regulation, we analyzed DNA methylation of the Terminal Inverted Repeats (TIRs). First, we used an assay that combines digestion of DNA using DNA methylation-sensitive restriction enzymes and quantitative PCR (Figure 5A). As controls, we used the methylation-insensitive enzyme DdeI and compared the mutants to nrpd1a (PolIVa) mutants, in which most of the asymmetric DNA methylation is lost [6,8]. Using three different enzymes that report on CNG and CHH sites (Figure 5A), we found a pronounced loss of DNA methylation in foe and in foe fca, foe fpa, foe fld and fpa fve (Figure 5B, C). Despite the stronger reactivation of AtMu1 expression in foe fpa compared to foe, DNA methylation levels in both mutants were similarly low (Figure 5C), suggesting that the further increase in expression was independent of DNA methylation. We confirmed the loss of DNA methylation at CNG and CHH sites in foe and in fpa using bisulfite sequencing of the AtMu1 TIR region (Figure 3D, Table S1). CG DNA methylation at AtMu1 was not or only slightly affected in foe and fpa foe.

During the double mutant expression analysis, we found that fca fld mutants had hyper-repressed AtMu1. Interestingly, this hyper-repression correlated with an increase in asymmetric DNA methylation in fca fld, but not fld single mutants (Figure 5B). Further studies will be necessary to understand the basis of this effect. At present, we can speculate that in the absence of FLD, an FCA-like protein can take its place; in the presence of FCA, this FLD-like protein would contribute to basal activation of AtMu1, whereas loss of FCA would cause this protein to become a strong repressor, possibly by switching its specificity to demethylate certain residues on histone tails. FLD homologues have been described recently [31], as has the context-dependent switch of specificity for the human FLD homologue, LSD1 [32,33].

Asymmetric DNA methylation is thought to be directed by sRNA [1,3,5]. We did not find a change in the abundance of sRNA at AtMu1, AtSN1 or IG/LINE (soloLTR) in any of the double mutants tested (Figure 6), suggesting that none of the AP genes play a role in the amplification of sRNA, but rather that they act either downstream or independent of sRNA. AtMu1 sRNA and asymmetric DNA methylation are lost in PolIVa mutants, yet expression increases only about 6-fold [8]. In contrast, we have shown here that AtMu1 expression in foe fpa increases ~70-fold, suggesting the involvement of DNA methylation-independent effects besides the observed reduction in DNA methylation.

Reactivation of transcription in the presence of DNA methylation has previously been reported for the targets of the MORPHEUS’ MOLECULE1 (MOM1) gene [34,35] and for AtSN1 in foe fpa [8]. Both MOM1 and FCA FPA act in parallel to DNA methylation, and loss of DNA methylation through mutation or application of the DNA methylation inhibitor 5-aza-deoxycytidine (aza-dC) in mom1 or fca fpa leads to dramatic developmental perturbations [6,34]. To find evidence for the DNA methylation-independent role of other components of the autonomous pathway, we tested whether any of the double mutants tested in this study showed hypersensitivity to aza-dC. Indeed, at aza-dC concentrations that did not affect development in wild type or fca or fpa single mutants, development in foe fpa and fpa fld mutant seedlings was strongly perturbed similar to what was reported for foe fpa (Figure 7, Table S2 and [8]). foe fpa and fpa fve were also hypersensitive to aza-dC, albeit to a slightly lesser extent (Figure 7). Together, our results demonstrate that AP components mediate silencing through both DNA methylation-dependent and -independent effects (Figure 6).

Conclusions

The autonomous pathway was initially identified as a flowering-specific pathway that promotes flowering by repressing expression of the floral repressor FLC. However, it is now clear that it has more widespread roles on other targets in the Arabidopsis genome [8,36]. Here, we have investigated how components of the autonomous pathway functionally interact to achieve this silencing.

Using gain-of-function analysis of the RRM-domain protein FPA to complement our loss-of-function double mutant analysis, we find that FPA at least partially acts through the histone demethylase FLD to repress FLC. Notably, FPA acts independently of the 3′-processing factor FY. This is in contrast to the other RRM-domain protein FCA, which acts through both FY and FLD. Thus, FCA and FPA have similar but distinct functions in repressing FLC. The MSI1 homologue FVE, in contrast, functions independently of FCA and FPA on FLC. However, analysis of the DNA transposon AtMu1 in double mutants and lines overexpressing FCA or FPA in an foe mutant background is consistent with the notion that on this target FCA acts through FVE.

Figure 6. Accumulation of sRNA from a range of targets (AtMu1, AtSN1, IG/LINE (solo LTR)) is not affected in AP double mutants. 9 μg of RNA enriched for the low molecular weight fraction from 14 day old seedlings of the indicated double mutants or nrpd1a was loaded per lane. Micro RNA mir171 is shown as a control.

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In general, we find that the effects of the AP genes and their interactions differ with each target analyzed and show no correlation with FLC levels, indicating the observed widespread effects are unlikely to be secondary effects of FLC overexpression. We therefore view the autonomous pathway not as a linear pathway, but rather a module of proteins whose role may be to recognize certain RNA features (presumably via the RNA-binding proteins) and trigger a reduction in the transcription of the corresponding loci (presumably via the chromatin regulators). This process is likely to be highly coordinated with transcription and transcript maturation (processing, capping, splicing). It is also possible that different modules of the autonomous pathway interact with different parts of the transcription and maturation machinery. We propose that the autonomous pathway is part of a widely conserved transcriptome surveillance mechanism and in Arabidopsis the gene encoding the flowering repressor FLC has, perhaps through selection for flowering time variation, become a very sensitive target.

**Materials and Methods**

**Plant Materials**

All mutants were in the Col background and have been described: fca-9, fpa-7, fpa-8 [8], fve-3 [16,17], fld-3, fld-4 [15], flk-1 [20], Col FRI Sf2 [37], mpd1-5 [38], ddm1-2 [39]. Plants were grown in long day conditions in soil at 23°C or on GM minus glucose plates at 20°C.

**Construction of 35S::FPA**

A genomic FPA fragment (coding sequence plus introns) was amplified with flanking BamHI sites [primers 30/FPA_BamHI_F (AAAGGATCCAGACTGCGTTATCTATGAAGCCATTC-]

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**Figure 7.** Several AP double mutants (fca fpa, fca fve, fpa fld, fpa fve, fpa flk) are hypersensitive to the DNA methylation inhibitor aza-dC. Seedlings were grown for 14 days on plates containing the indicated concentration of aza-dC before their phenotypes were scored. (A) A representative seedling of the indicated genotypes and treatments is shown. All pictures are the same magnification and represent 15 mm × 15 mm original size. (B) Seedlings were grouped into different classes based on the phenotype of their primary leaves (fully expanded leaves, only 1 leaf expanded, leaves bit expanded stub/stump/pin, no leaves). Severity of the phenotypes increased with increasing aza-dC concentration. doi:10.1371/journal.pone.0002733.g007
AGAGC] and 31/FPA_BamHI_R (AAGAGCATCTCAAGGC-
CCCTGTCCAGCCGGAGTA) and inserted into 35S::pBIN-
Plus [40].

RNA and DNA methylation analysis
RNA was extracted from 14 day old seedlings and analyzed as described [8]. Bisulfite sequencing was performed as described [8]. For determining DNA methylation through quantitative PCR, we extracted DNA from 14 day old seedlings using the QIAGEN DNeasy Plant Mini Kit and digested 20 ng of DNA overnight with 15 units of the indicated restriction enzyme. After inactivating the restriction enzyme, we immediately performed quantitative PCR using 0.3 ng of DNA per PCR reaction and primers 96/
MuTIR_F and 97/MuTIR_R as described in [8]. Primers for FLC quantitative RT-PCR were FLC_cDNA_393F (AGCCAAGAGGAGC) and 31/FPA_BamHI_R (AAAGGATCCTCAAGGC-
CCCTGTCCAGCCGGAGTA) and FLC_cDNA_530R (TTTGTCCTACCGAGGTGACATC). All other primers have been described [8].

Supporting Information
Table S1 Additional information for Bisulfite sequencing of AtMu1.
Found at: doi:10.1371/journal.pone.0002733.s001 (0.04 MB DOC)
Table S2 Additional information on the Percentages of abnormal seedlings after 14d growth on the indicated concentration of aza-dC.
Found at: doi:10.1371/journal.pone.0002733.s002 (0.10 MB DOC)

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Author Contributions
Conceived and designed the experiments: IB CD. Performed the experiments: IB CD. Analyzed the data: IB. Wrote the paper: IB CD.

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