Antagonistic Actions of FPA and IBM2 Regulate Transcript Processing from Genes Containing Heterochromatin

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Repressive epigenetic marks, such as DNA and histone methylation, are sometimes located within introns. In Arabidopsis (Arabidopsis thaliana), INCREASE IN BONSAI METHYLATION2 (IBM2), an RNA-binding protein containing a bromo-adjacent homology domain, is required to process functional transcript isoforms of genes carrying intronic heterochromatin. In a genetic screen for suppressors of the ibm2 mutation, we identified FPA, an RNA-binding protein that promotes use of proximal polyadenylation sites in genes targeted by IBM2, including IBM1 encoding an essential H3K9 histone demethylase and the disease resistance gene RECOGNITION OF PERONOSPORA PARASITICA7. Both IBM2 and FPA are involved in the processing of their common mRNA targets: Transcription of IBM2 target genes is restored when FPA is mutated in ibm2 and impaired in transgenic plants overexpressing FPA. By contrast, transposons targeted by IBM2 and localized outside introns are not under this antagonistic control. The DNA methylation patterns of some genes and transposons are modified in fpa plants, including the large intron of IBM1, but these changes are rather limited and reversed when the mutant is complemented, indicating that FPA has a restricted role in mediating silencing. These data reveal a complex regulation by IBM2 and FPA pathways in processing mRNAs of genes bearing heterochromatic marks.

DNA and histone methylations are epigenetic marks found in plants and animals that influence chromatin structure and have a direct impact on gene function and transposon mobilization. Chromatin can be modified and remodeled in several ways. In plants, the Jumonji-C domain-containing protein INCREASE IN BONSAI METHYLATION1 (IBM1) is a histone demethylase that removes methylation on Lys-9 of histone H3 (H3K9me). IBM1 function is essential in plants because it prevents deposition of these heterochromatic silencing marks at transcribed genes (Saze et al., 2008; Miura et al., 2009; Inagaki et al., 2010). H3K9me and CHG DNA methylation (where H = A, T, or C) are tightly correlated. Indeed, CHG methylation is controlled by the DNA methyltransferase CHROMOMETHYLASE3 (CMT3) recruited to regions enriched in H3K9me, which it directly binds (Du et al., 2012, 2014). In a reciprocal manner, H3K9me is catalyzed by three histone methyltransferases, SU(VAR)3-9 HOMOLOG4/KRYPTONITE (SUHV4/KYP), SUHV5, and SUHV6 (Ebbs and Bender, 2006). KYP binds CHG-methylated cytosines through its SRA domain (Johnson et al., 2007). Thus, CMT3 and KYP participate in a self-reinforcing loop between DNA and histone methylation, which is needed for silencing transposons and repeat sequences but is deleterious to genes when IBM1 is absent. Consequently, ibm1 mutants accumulate both H3K9me and CHG in coding regions with drastic consequences for development (Saze et al., 2008; Miura et al., 2009).

Two other categories of Arabidopsis (Arabidopsis thaliana) mutants share the ibm1 developmental and molecular phenotype: mutants of the IBM2/ANTI-SILENCING1/SHOOT GROWTH1 gene, hereafter called IBM2 (Saze et al., 2013; Wang et al., 2013; Coustham et al., 2014) and mutants of ENHANCED DOWNY MILDEW2 (EDM2; Tsuchiya and Eulgem, 2013a). The IBM1 gene encodes
two different transcripts of which only the longest encodes a functional protein (Rigal et al., 2012), and its production is controlled by both IBM2 and EDM2 in a yet unclear manner. IBM2 is a protein of unknown function containing a bromo-adjacent homology domain and an RNA-recognition motif (Saze et al., 2013; Wang et al., 2013; Coustham et al., 2014). EDM2 contains several zinc-finger domains and a region similar to the active domains of certain methyltransferases (Tsuchiya and Eulgem, 2013b). Both EDM2 and IBM2 are found in the same protein complex, bridged by the AS11-IMMUNOPRECIPITATED PROTEIN1 (AIPP1; Duan et al., 2017). In addition to the IBM1 gene, EDM2, AIPP1, and IBM2 share another target, the disease resistance gene RECOGNITION OF PEROXOSPORA PARASITICA7 (Saze et al., 2013; Tsuchiya and Eulgem, 2013a; Wang et al., 2013; Lai et al., 2018). Like IBM1, RPP7 contains a heterochromatic domain within a long (>2 kb) intron associated with H3K9me and DNA methylated in all cytosine contexts. The IBM2 complex associates with these methylated intronic regions (Saze et al., 2013; Tsuchiya and Eulgem, 2013a; Wang et al., 2013) to produce the full-length functional transcript by an unknown molecular mechanism. One hypothesis is that EDM2/AIPP1/IBM2 function by enhancing the use of distal polyadenylation sites over proximal sites located in large introns.

Polyadenylation is one key mRNA processing step, and the choice between alternative polyadenylation sites impacts the regulation of gene expression. FPA is a RNA-binding protein with three RNA-recognition motifs involved in polyadenylation site choice; it plays a major role in repressing floral transition by favoring the proximal polyadenylation site of an antisense of the FLOWERING LOCUS C transcript (Hornyik et al., 2010a; Liu et al., 2010) and more broadly in regulating the 3′-end site choice of diverse mRNAs (Sonmez et al., 2011; Duc et al., 2013), including its own transcript (Macknight et al., 2002; Hornyik et al., 2010b). So far, FPA has not been identified as a member of any splicing or polyadenylation complexes, and the precise function of FPA and its mode of action are still unclear. fpa mutants have also been identified in a genetic screen aimed at finding components required for RNA-mediated chromatin silencing (Bäurle et al., 2007), but the role played by FPA in silencing has not been explored. In addition, FPA is involved in plant defense responses (Lyons et al., 2013), pointing toward a more general role in addition to flowering.

Here, we identify fpa as a suppressor of the ibm2 phenotype. The transcription of both IBM1 and RPP7 is restored in a double fpa ibm2 mutant and impaired when FPA is overexpressed. We show that fpa mutants are depleted in CHG methylated cytosine within the largest introns of IBM1, providing evidence that mutating FPA has an effect on chromatin structure that nevertheless seems to be limited to specific regions. We demonstrate that RNA-binding proteins, like FPA and IBM2, are involved in an intricate crosstalk between chromatin and RNA processing to regulate the production of key genes such as IBM1 and RPP7. We further show that transposons controlled by IBM2 localized outside introns are unaffected by this mechanism.

RESULTS

fpa Is a Genetic Suppressor of ibm2

To uncover new genes impacting the function of IBM2, we performed a forward genetic screen to isolate suppressors of ibm2-4, an allele previously called sg1-1 (Coustham et al., 2014). Approximately 7,000 ibm2-4 seeds were treated with ethylmethane sulfonate (EMS; see “Materials and Methods”). The genetic screen was performed in two steps on 88,000 M3 seedlings. Because the ibm2 phenotype is related to a deficiency in production of the long functional IBM1 mRNA (Saze et al., 2013; Wang et al., 2013; Coustham et al., 2014), we screened for mutants showing a wild-type phenotype, aiming to select suppressors in which the function of IBM1 was restored. We postulated that this pool of plants contained suppressors of ibm2, but also suppressors restoring the effects of a nonfunctional IBM1. Mutations like cmt3 or kyp, for instance, suppress ibm1 by preventing the accumulation of heterochromatic marks on a large range of IBM1 targets (Saze et al., 2008). These mutations did not restore the transcription of IBM2 targets such as AT3G05410 or RPP7 (Supplemental Fig. S1). Next, to isolate genetic suppressors of ibm2 more specifically from the first screen, we determined the level of transcription of AT3G05410 in M3 plants (from the same M2 pool) that resembled wild-type plants but had more serrated leaves (Fig. 1A) and were late-flowering (Supplemental Fig. S2). In these plants, mRNA levels of the known IBM2 target AT3G05410 were intermediate between ibm2-4 and wild type (Supplemental Fig. S3). Therefore, the three mutants are likely progeny from the same M2 plant. By sequencing and comparing the genomes of these plants and the original ibm2-4 mutant, we identified mutations that were homozygous and common to the three plants but not the original mutant. Sequencing revealed that the suppressor of ibm2-4 carries a nucleotide change (C-to-T) in the fifth exon of FPA (AT2G43410) at position 586, creating a premature stop codon. The ibm2-4 suppressor was therefore designated a new fpa allele (fpa-11). These results show that fpa is epistatic over ibm2.

We then extracted genomic DNA from leaves of fpa-11 ibm2-4 mutants to perform whole-genome bisulfite single-base resolution sequencing (Supplemental Table S1) and determine the patterns of methylation for genes. On average, both CG and CHH methylation levels were similar in genes of ibm2-4 and fpa-11 ibm2-4 mutants (Supplemental Fig. S4); however, the CHG hypermethylation accumulating in ibm2 genes (Saze et al., 2013; Wang et al., 2013; Coustham et al., 2014) was lost in genes of fpa-11 ibm2-4 (Fig. 1B). We confirmed the results by identifying the Differentially Methylated Regions (DMRs) in ibm2-4 or fpa-11 ibm2-4
also detected a general increase of double fpa ibm2 mutants. The average methylation levels of genes were determined by dividing the genes into 100-bp bins. Regions located 1 kb upstream and 1 kb downstream are shown. C. Total number of DMRs found in the three methylation contexts (mCG, mCHG, and mCHH). Hypo- and hypermethylated DMRs are shown.

Figure 1. Phenotype of the ibm2 suppressor. A, Wild-type (Col-0), ibm2-4, and the ibm2 suppressor (fpa-11 ibm2-4) plants were grown in the greenhouse and pictured after 25 d. Scale bar = 1 cm. B, CHG methylation levels of genes in ibm2-4 and fpa-11 ibm2-4 mutants. The average methylation levels of genes were determined by dividing the genes into 100-bp bins. Regions located 1 kb upstream and 1 kb downstream are shown. C, Total number of DMRs found in the three methylation contexts (mCG, mCHG, and mCHH). Hypo- and hypermethylated DMRs are shown.

compared to wild type (Fig. 1C). Indeed, the number of ibm2 CHG hyperDMRs (n = 4722) was reduced by 35-fold in fpa-11 ibm2-4, implying that mutating FPA in ibm2 suppresses the CHG hypermethylation in genes.

Next, we quantified mRNA levels of known IBM2 targets (Supplemental Fig. S5) in fpa and fpa ibm2 mutants grown in vitro for 21 d. Reverse transcription quantitative PCR (RT-qPCR) analyses revealed that the IBM1-L transcripts were more abundant compared to wild type in all fpa allelic backgrounds tested, including fpa-3, which is a previously described allele (Hornyik et al., 2010b), fpa-11, fpa-3 ibm2-4, and fpa-11 ibm2-4 (Fig. 2; IBM1-L). The opposite trend was observed for IBM1-S (Fig. 2; IBM1-S). We also detected a general increase of IBM1 transcripts in fpa mutants using a set of primers amplifying all IBM1 transcript isoforms (Fig. 2; IBM1-total). Together, the expression data show that mutating FPA in both ibm2 and wild-type plants increased IBM1 transcripts by ~1.8-fold. Furthermore, in fpa backgrounds, the production of the long IBM1 transcript is favored over the shortest one. Levels of two other IBM2 targets (AT3G05410 and AT1G11270) were restored to 40% of wild type in the double fpa ibm2 mutants (Fig. 2; AT3G05410-L and AT1G11270-L). Finally, RPP7 (AT1G58602) mRNA levels were restored to 80% of wild type in the suppressor fpa ibm2 backgrounds (Fig. 2; RPP7-L). Altogether, the genetic screen for ibm2 suppressors, the methylene sequencing of fpa ibm2 mutants, and the RT-qPCR analyses of IBM2 targets demonstrate that a mutation in fpa counterbalances the absence of IBM2 by restoring the production of its target transcripts.

FPA Contributes to Processing of IBM2 Target Genes Containing Intronic Heterochromatin

To understand better the links between FPA and the processing of IBM2 targets, the levels of their transcripts were monitored when FPA was overexpressed. Compared to wild type, the production of IBM1-L, RPP7-L, AT1G11270-L, and AT3G05410-L mRNAs was reduced by 29%, 60%, 47%, and 54%, respectively, in plants expressing 35S:FPA-YFP constructs in a fpa-8 background (Fig. 3; Supplemental Fig. S6). Therefore, the long RPP7, IBM1, AT1G11270, and AT3G05410 transcripts are produced incorrectly in these transgenic plants, confirming the role played by FPA in their processing.

Next, we assessed the function of the Col-0 RPP7 gene in race-specific disease resistance against the biotrophic oomycete Hyaloperonospora parasitica isolate Hiks1 (Hpa Hiks1; Slusarenko and Schlaich, 2003). The triple suvh456 mutant, which has lost the RPP7 intragenic methylation, and two ibm2 alleles (ibm2-1 and ibm2-4) displayed reduced RPP7 resistance, indicated by increased growth of Hpa Hiks1 in leaves (Fig. 4), whereas fpa-3 and fpa-11 mutants were as resistant as Col-0 (Fig. 4). In agreement with the partial restoration of RPP7-L transcript levels in fpa-11 ibm2-4 (Fig. 2), this double mutant also exhibited partially restored RPP7-mediated resistance (Fig. 4). Therefore, FPA controls the resistance function of RPP7.

Direct RNA sequencing (DRS) helps to define polyadenylation sites by direct sequencing of RNAs in the absence of reverse transcription and is therefore a method of choice to localize regions where FPA promotes polyadenylation. We used the DRS data published previously for fpa mutants to identify polyadenylated 3' ends in fpa-7 and Col-0 (Duc et al., 2013). We found that distant polyadenylation sites of AT3G05410, IBM1, and RPP7 were more frequently used in fpa mutants at the expense of proximal polyadenylation sites (Fig. 5). Indeed, the number of normalized DRS reads corresponding to distal polyadenylated sites increased in the fpa-7 background by 2.4- to 1.9-fold.
Intronic DNA Methylation of IBM1 Decreases in fpa

The genes targeted by IBM2 contain introns carrying heterochromatic marks that regulate their transcription. Because FPA was previously identified in a mutant screen for genes required for the silencing of an inverted repeat (Baurle et al., 2007), we tested whether the DNA methylation patterns of IBM2 targets were modified in an fpa background. For this, we monitored methylation levels of the large IBM1 intron in the fpa mutants. After bisulfite conversion, we sequenced the corresponding IBM1 region in ibm2-4, fpa-11 ibm2-4, and fpa-3. Compared to wild type or to ibm2 controls, CHG methylation

1.7-fold for IBM1, AT3G05410, and RPP7, respectively. By contrast, the number of DRS reads corresponding to proximal sites decreased by 28.8- and 3.4-fold for IBM1 and AT3G05410, respectively. Hence, polyadenylation of IBM2 targets is mediated by FPA. The data indicate that FPA and IBM2 pathways are interconnected in processing of their common targets, including IBM1 and RPP7 transcripts.

IBM2 promotes the transcription of the methylated Copia element AT4G16870 (Duan et al., 2017), which is a nonintronic transposon localized upstream of the RPP4-resistant gene (Fig. 6A). A chimeric RPP4-AT4G16870 mRNA consisting of both RPP4 and this Copia element (Wang and Warren, 2010) was detected in the wild type but not in ibm2-4, aipp1-1, edm2-4, or swkh456 (Fig. 6B; Supplemental Fig. S7A), confirming that the transcription of RPP4-AT4G16870 is promoted by an EDM2/AIPP1/IBM2 complex and relies on the presence of heterochromatic marks controlled by SUVH proteins. Similarly, RPP4-AT4G16870 mRNAs were not detected in fpa-11 ibm2-4 mutants but were expressed in fpa-11 (Fig. 6B). Thus, the loss of FPA does not restore the transcription of RPP4-AT4G16870 mRNAs in ibm2. We verified that the RPP4-mediated resistance to Hpa isolate EMWA1 was not compromised in ibm2, edm2, fpa ibm2, or fpa mutants (Supplemental Fig. S8). To identify additional nonintronic IBM2 targets, transposons differently expressed in ibm2, edm2 and aipp1, compared to wild-type plants, were listed using published RNA-seq data (Duan et al., 2017). We found a total of 18 transposons that were significantly (false discovery rate threshold ≤ 0.05) downregulated (log2FC[ibm2 and edm2 and aipp1/wild type] < −2) in ibm2, edm2, and aipp1 (Supplemental Table S2). Six transposons corresponded to known IBM2 targets like the intronic RPP7 transposons or the Copia element AT4G16870. In addition, we found that AT4TE21110 is another nonintronic IBM2 target expressed in Col-0 and fpa mutants but not in ibm2 or fpa ibm2 mutants (Fig. 6C; Supplemental Fig. S7B), as observed for RPP4-AT4G16870. Altogether, our data show that FPA and IBM2 pathways are antagonistic at genes containing heterochromatin within their introns, like IBM1 or RPP7, but not at nonintronic IBM2 targets such as the Copia element AT4G16870 or AT4TE21110.

Figure 2. Expression analyses of IBM2 target genes in fpa mutants. The expression of IBM2 targets were determined by RT-qPCR in ibm2-4, fpa-3, and fpa-11 back-crossed twice to Col-0 and the double ibm2-4 fpa-11 and ibm2-4 fpa-3 mutants. Results were normalized to Col-0 (expression fixed at “1” for each experiment). The PCR fragments amplified are shown in Supplemental Figure S5. Error bars represent so (n = 9). The asterisks indicate a significant difference between the sample and the corresponding Col-0 control determined by Student’s t test (*P < 0.05; **P < 0.01; ***P < 0.001).
was reduced by almost half in the fpa-11 ibm2-4 and the fpa-3 mutants (Fig. 7A; Supplemental Fig. S9). We examined the methylation patterns of the same IBM1 region in mutants for which the whole methylomes were sequenced (Stroud et al., 2013), and we found reduced CHG methylation in both fpa-7 and fca-9 fpa-7 plants (Supplemental Fig. S10). Therefore, the methylation of IBM1 is modified in fpa backgrounds.

To explain the reduction of CHG methylation observed at IBM1 in fpa, we hypothesized that IBM1 could...
control the production of its own mRNA by removing intronic epigenetic marks contained within the largest intron of the IBM1 gene. If this hypothesis was correct, increased levels of IBM1-L transcript—as observed in fpa—would result in the demethylation of the IBM1 intron. To test whether such a feedback loop exists, we monitored patterns of DNA methylation in the intron of IBM1 when IBM1-L was overexpressed ectopically (Supplemental Fig. S6). Indeed, IBM1 controls methylation of H3K9, which cross-regulates the levels of mCHG (Johnson et al., 2007; Du et al., 2012, 2014). Our data show that cytosine methylation levels of the IBM1 intron were comparable between wild type and ibm1 mutants that overexpress the IBM1-L complementary DNA (cDNA; Fig. 7A; Supplemental Fig. S9). As the methylation patterns of the IBM1 intron remained unchanged when IBM1-L was more abundant, we concluded that the hypomethylation of IBM1 in fpa is likely not associated with the increased production of IBM1-L transcripts observed in this mutant background.

**The Absence of FPA Induces Transient Methylation Changes**

To understand whether the decrease of methylation we observed for IBM1 was widespread or limited to specific regions of the genome, we sequenced the whole methylene of fpa mutants. Levels of methylation per cytosine confirmed that biological replicates were closely correlated (pairwise Pearson correlation values between biological replicates 0.97 for CGs, 0.98 for CHG, and 0.94 for CHH). When the average methylation levels were calculated in 100-bp windows partitioning the genome, we observed no broad changes between wild-type plants and fpa-3 (Fig. 8A), confirming results obtained with the fpa-7 T-DNA allele (Stroud et al., 2013). Next, we identified the DMRs in fpa-3 and compared them to the wild type and to the transgenic fpa-8 line complemented by a 35S:FPA-YFP construct (Bäurle et al., 2007) that was also sequenced. The spontaneous DMRs naturally occurring within the Arabidopsis Col-0 accession were filtered (Zhang et al., 2018). We identified 61 CG hypoDMRs, 73 CG hyperDMRs, seven CHG hypoDMRs, two CHG hyperDMRs, two CHH hypoDMRs, and six CHH hyperDMRs arising in fpa-3 and returning to wild-type methylation patterns when the function of FPA was restored (Supplemental Table S3). Most of the CG hyperDMRs were found in genes (Fig. 8B) and were de novo-methylated in fpa (Fig. 8C; CGhyper), while CG hypoDMRs overlapped with transposons (Fig. 8B) that were demethylated in fpa (Fig. 8C; CGhyp). The IBM1 intronic region carrying the heterochromatic marks (Fig. 7B) was identified among the seven CHG hypoDMRs, confirming the results obtained by targeted bisulfite sequencing (Fig. 7A; Supplemental Fig. S9). A limited number of other regions remained differentially methylated in both fpa-3 and the fpa complemented line compared to their respective wild-type
controls: 12 CG hypoDMRs, nine CG hyperDMRs, two CHG hypoDMRs, two CHG hyperDMRs, and one CHH hypoDMR. Therefore, most of the changes of methylation patterns in fpa, including those at IBM1, are reversible when the mutant is complemented by a construct overexpressing FPA.

Next, we examined the methylation profiles of transposable elements that are derepressed in fpa. Previous studies have revealed that transposons like AtSN1, which is a SINE retroelement; AtMu1, which is a DNA transposon; and the helitron ATITE93275 are expressed in the fpa backgrounds in contrast to wild type (Bäurle et al., 2007; Sonmez et al., 2011). No changes in DNA methylation were observed for AtMu1 in fpa-3 or for AtSN1 (Supplemental Fig. S11). We confirmed that ATITE93275 is demethylated in all cytosome contexts in fpa-3 (Supplemental Fig. S11), matching one of the four CHG DMRs that remained hypomethylated when fpa was complemented. These results indicate that some transposons, which are upregulated in fpa, are associated with differences in DNA methylation patterns, but their number is low because we found no widespread changes of transposon methylation patterns in fpa.

DISCUSSION

Here we identify fpa as a genetic suppressor of the ibm2 mutation. The IBM2 protein complex interacts with heterochromatic marks localized within the large introns of IBM2 target genes to promote production of their long transcripts. FPA, by promoting polyadenylation of shorter transcripts, antagonizes the function of IBM2. In addition, FPA affects methylation of the largest intron of IBM1 and a limited number of other regions.

Crosstalk between FPA and IBM2 in Polyadenylation Site Choice

Our forward genetic screen to isolate suppressors of ibm2 revealed that FPA is involved in the processing of IBM2 targets. Levels of AT1G11270 and AT3G05410 long transcripts were restored to 40% of wild-type levels in a double fpa-11 ibm2-4 mutant, and levels of RPP7 long mRNAs were restored to 80% (Fig. 2; RPP7-L). In addition, IBM1 long mRNAs were ~1.8-fold more abundant in fpa compared to wild type (Fig. 2; IBM1-L). Consequently, we found that the most distant polyadenylation sites of IBM2 target genes are favored in an fpa background (Fig. 5). At the same time, the large intron of IBM1 contained less methylation in fpa mutants (Fig. 7; Supplemental Fig. S9; Supplemental Table S3), although both CHG and H3K9 methylation within introns appear to be crucial for processing IBM2 target transcripts. First, these transcripts are incorrectly processed when intronic transposons are depleted of CHG methylation (Le et al., 2015). Indeed, RT-qPCR analyses revealed that levels of long RPP7 and AT3G05410

Figure 6. Expression analysis of IBM2 nonintronic target transposons. A, Schematic representation of the Copia (AT4G16870-RPP4 (AT4G16860)) locus targeted by IBM2. The exons of RPP4 are in blue, and the Copia element is in green. B, Expression analysis of RPP4, AT4G16870, and the chimeric RPP4-AT4G16870 transcripts in Col-0 and different mutant backgrounds. cDNAs were amplified using primers indicated in (A) and described in Wang and Warren (2010). ATEF cDNA amplifications served as controls. C, Expression analysis of AT4TE21110 in Col-0 wild type and different mutant backgrounds. AT4TE21110 is localized in the pericentromeric region of chromosome 4. RNAs were extracted from bulks (#1 and #2) of 20 plants grown in vitro for 15 d and cDNAs were amplified using primers described in Supplemental Table S4. ACTIN cDNA amplifications served as controls.
transcripts are reduced in cnt3 (Le et al., 2015), confirming results obtained for IBM1 long transcripts (Rigal et al., 2012). Second, compromising the functions of H3K9 histone methyltransferases has similar consequences. Northern-blot analyses show that kyp/suvh4 mutants produce lower levels of IBM1 long transcripts compared to wild type (Rigal et al., 2012). If methylation marks are necessary to correctly transcribe IBM2 target genes, how then can IBM2 targets be transcribed in an fpa background in which intragenic heterochromatic marks are reduced? Because fpa is epistatic to ibm2, it is likely that IBM2 is important for the production of its target transcripts only when FPA is functional. A possible explanation is that FPA promotes the recruitment of the polyadenylation complex at proximal sites, while IBM2 antagonizes this binding. We suggest that IBM2 prevents the polyadenylation of short transcripts only when FPA is active. Whether IBM2 and/or its partners interact directly with proteins of the polyadenylation complex remains to be determined. This mechanism might apply to other plant species. In oil palm (Elaeis guineensis), for instance, the transcription of an essential homeotic gene is regulated by methylation of an intronic LINE retroelement (Ong-Abdullah et al., 2015). Levels of transcription also correlate with the size of heterochromatic regions. Expression of genes with long methylated introns, such as RPP7, AT3G05410, or AT1G11270, is decreased by >70% in ibm2 and partially rescued in fpa ibm2 (Fig. 2).

However, the transcription of IBM1, which contains a shorter methylated intronic region, is decreased by only 50% in ibm2 and restored in fpa ibm2 (Fig. 2) in which IBM1 is fully functional (Fig. 1, B and C). By inserting into introns, transposons introduce alternative polyadenylation sites, making the targeted gene regulated by both the IBM2 and FPA pathways. By contrast, FPA does not antagonize IBM2 for IBM2 transposon targets localized outside genic regions (Fig. 6).

Links between RNA Processing and Methylation Changes

The role played by FPA in controlling silencing remains controversial. The fpa mutants were first retrieved from a forward genetic screen to identify genes involved in RNA silencing (Bäurle et al., 2007). Even if some transposons are strongly reactivated in fpa (Bäurle et al., 2007; Sonmez et al., 2011), the analysis of DRS data showed no widespread differences of expression for transposons between fpa-7 and the wild type (Duc et al., 2013). In addition, fpa-7 methylation analyses revealed no major differences in methylation patterns (Stroud et al., 2013). fpa-7 is a transfer DNA (T-DNA) allele, and recent studies have demonstrated that fpa mutations can rescue T-DNA insert mutants (Zhang et al., 2016), possibly explaining the phenotypic discrepancies existing between T-DNA and point mutation fpa alleles (Duc et al., 2013). By sequencing the
When the function of FPA is restored, indicating that the changes of methylation are rather limited and transient. Mutating FPA more specifically disturbs DNA methylation of heterochromatic regions localized within the largest intron of IBM1 in contrast to other IBM2 targets. By sequencing the methylome of fpa-3, the largest intron of IBM1 was identified as a CHG hypomethylated DMR (out of seven in total; Supplemental Table S3), confirming results obtained by targeted bisulfite sequencing in fpa-11 ibm2-4 (Fig. 7A). Likewise, the CHG methylation, localized at the endogenous phytoene desaturase locus silenced by an inverse repeat introduced transgenically in trans, is compromised in the fpa-8 background (Bäurle et al., 2007). Hypomethylation of IBM1 observed in fpa is probably not coupled to an increase of IBM1-L production, because transgenic ibm1 plants overexpressing IBM1-L ectopically show no alteration of methylation at IBM1 (Fig. 7A; Supplemental Fig. S9). Moreover, the limited number of CHG DMRs found in an fpa background (Supplemental Table S3) argue against FPA directly controlling the activity of CMTs at transposons localized near or within genes. Therefore, the loss of methylation at IBM1 in fpa is probably independent of IBM1 activity. Other factors might account for this hypomethylation, such as those associated with the changes of polyadenylation site in fpa mutants and the subsequent effects on IBM1 mRNA processing. Previous studies have demonstrated that transcription initiation and/or the polymerase II elongation rates are influenced by choice of polyadenylation sites at FLOWERING LOCUS C (Wu et al., 2016). Similarly, polymerase II occupancy is increased near the proximal polyadenylation sites of RPP7 in edm2, app1, and suvh456 plants, indicating that the enzyme is probably pausing in this region (Lai et al., 2018). Therefore, changes of polyadenylation sites in fpa also very likely modify the rates of transcription for many genes, including IBM1 and transposons, which might impact their methylation patterns. Furthermore, the recent discovery that human RBM15 proteins, related to FPA, direct methylation to promoters and provide evidence that they act antagonistically controlling the transcription of their common targets RPP7 and IBM1-L, partially disturbs DNA methylation of heterochromatic regions localized within the largest intron of IBM1 and accumulate CHG DMRs identified near or within genes, therefore the loss of methylation at IBM1 in fpa is probably independent of IBM1 activity. Other factors might account for this hypomethylation, such as those associated with the changes of polyadenylation site in fpa mutants and the subsequent effects on IBM1 mRNA processing. Previous studies have demonstrated that transcription initiation and/or the polymerase II elongation rates are influenced by choice of polyadenylation sites at FLOWERING LOCUS C (Wu et al., 2016). Similarly, polymerase II occupancy is increased near the proximal polyadenylation sites of RPP7 in edm2, app1, and suvh456 plants, indicating that the enzyme is probably pausing in this region (Lai et al., 2018). Therefore, changes of polyadenylation sites in fpa also very likely modify the rates of transcription for many genes, including IBM1 and transposons, which might impact their methylation patterns. Furthermore, the recent discovery that human RBM15 proteins, related to FPA, direct methylation to specific noncoding RNAs (Patil et al., 2016), is consistent with a role for FPA in mRNA methylation, implying that a previously unrecognized interplay exists between epigenetic silencing marks and methylation of IBM2 target RNAs.

**CONCLUSION**

We show that FPA and IBM2 pathways are crucial for controlling the transcription of their common targets and provide evidence that they act antagonistically when transposons are inserted in introns. The tight regulatory control of RPP7 mRNA levels is likely critical to limit the accumulation of a long functional RPP7 transcript in pathogen-unchallenged conditions and to prevent autoimmunity. In the presence of Hpa and Hiks1, the fine regulation of RPP7 transcripts mediated by both IBM2 and FPA favors accumulation of a long methylome of the point mutation fpa-3 allele, we confirmed that no major changes of DNA methylation patterns were observed genome-wide (Fig. 8A), but we found that CG methylation was gained in some genes and lost in some transposons of fpa (Fig. 8, B and C). Most of these changes revert to wild-type patterns

![Figure 8](https://plantphysiol.org)
transcript to induce a rapid and specific immune response (Tsuchiya and Eulgem, 2013a). Similarly, the importance of H3K9 methylation in resistance to viruses has been described (Sun et al., 2015), and the virulence of geminiviruses requires a viral protein that inhibits expression of the main plant H3K9 methyltransferase, KYP. To reinforce the action of KYP, rapid modulation of IBMI gene expression, mediated by both FPA and IBM2, is likely essential. Our data suggest that intrinsic heterochromatic marks associated with alternative polyadenylation sites can be decoded by RNA-binding proteins like FPA and IBM2 to tune the expression of key regulator genes such as IBMI or RPP7.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) accession Ksk-1 was described in Slusarenko and Schlaich (2003). All other plants were in the Arabidopsis Col-0 background. The ibm2-4 point mutation was previously named sig-1, and ibm2-5 (SAIL_31B006) was sig-2 (Coustham et al., 2014). The following mutants were previously described: atsn2-4, SAILK_142563 (Eulgem et al., 2007), fpa-3 (Hornyk et al., 2010b), fpa-7 (Michaels and Amasino, 2001; Veleys and Michaels, 2008), fpa-8 (Baurle et al., 2007), ibm1-1 (Saże et al., 2008), ibm2-1 (Saże et al., 2013), the triple sauvk56 mutant (Ebbs and Bender, 2006), cm3-11 ibm2-5 (Coustham et al., 2014), and kyp ibm2-5 (Coustham et al., 2014). The following lines were previously described: the transgenic fpa-8 line (Col-0 background) carrying a 35S:FP-A-YFP construct (Baurle et al., 2007) and the ibm1-3 complemented lines expressing a 35S:FPA-IBM1-c cDNA (Fan et al., 2012).

For EMS mutagenesis, ~7,000 ibm2-2 seeds were incubated in water containing 0.1% (v/v) EMS for 15 h at room temperature and washed several times with water. Plants were then grown in pools of 16 (440 M1 pools in total) in greenhouses in long-day conditions at 20°C. The next generation was obtained with water. Plants were then grown in pools of 16 (440 M1 pools in total) in growth chambers at 21°C in long-day conditions.

Gene Expression Analyses

Total RNA was isolated from the aerial parts of 21-d-old seedlings grown in vitro using the RNeasy Plant Mini kit (Qiagen) followed by a DNAse treatment (Fermentas). RT-PCR was performed on 500 ng (except for fpa amplification where 1 µg was used) of total RNAs with the M-MLV reverse transcriptase (Fermentas), and cDNAs were diluted 10 times. Five µl was used for RT-qPCR using a CFX96 real-time PCR machine (BioRad) with a SYBR solution (Eurogentec) and primers listed in Supplemental Table S4. Expression levels were normalized against the Arabidopsis UBC21 gene (ATSG25760).

Pathogen Infection Assays

To test RPP7 disease resistance function, plants were inoculated with Hpa isolate Hiks1, which is an oomycete pathogen specifically recognized by RPP7 in Arabidopsis accession Col-0 and virulent on accession Ksk-1 (Slusarenko and Schlaich, 2003). The function of another Hpa resistance gene, RPP4, was assessed by inoculating the plants with Hpa isolate EMWA1, which is specifically recognized by RPP4 in Arabidopsis accession Col-0 and virulent on the Col-0 dsl1-2 mutant (Garcia et al., 2010). Briefly, 14-d-old plants were sprayed with water containing 4 × 105 Hpa Hiks1 spores per mL. Plant cell necrosis and Hiks1 or EMWA1 hyphal development were monitored by staining leaves with lactophenol trypan blue as described in Koch and Slusarenko (1990). Stained leaves were viewed under a binocular light microscope. Infection assays were repeated independently at least three times with similar results.

Whole-Genome Sequencing and Bioinformatic Analyses

The genomes of fpa-11 ibm2-4 and ibm2-4 were sequenced using HiSeq technology (Illumina). Mutations were identified using the MutDetect pipeline described in Girard et al. (2014).

For fpa-3, fpa-8 35S:FPA-YFP, and fpa-11 ibm2-4 methylome sequencings, bisulfite treatment, library preparation, and whole-genome sequencing (final depth of 20X) were performed by the Beijing Genomics Institute using HiSeq technology (Illumina) producing 100-bp paired-end reads (Supplemental Table S1). Reads were trimmed with Trim_Galore (Babraham Bioinformatics) and aligned to the Col-0 Arabidopsis (The Arabidopsis Information Resource [TAIR10]) reference genome with the software Bismark (version 0.14.5; Babraham Bioinformatics) using standard options (Bowtie2; one mismatch allowed). Identical pairs were collapsed. Subsequent analyses were done using the following R packages: bseq version 1.7.7 (Hansen et al., 2012) and DSS version 2.11.3 (Wu et al., 2015) to call DMRs as described in Corem et al. (2018). The hcDMR pipeline was used as indicated by Zhang et al. (2018) to filter spontaneous DMRs occurring in Arabidopsis Col-0 bisulfite sequencings. DMRs arising in fpa and restored to wild-type patterns in fpa complemented lines corresponded to DMRs found between fpa-3 and the corresponding Col-0 controls that overlapped with DMRs found between fpa and the fpa complemented line but not with DMRs found between the fpa complemented line and its Col-0 control. DMRs that remained differentially methylated when the FPA function was restored corresponded to DMRs found between fpa-3 and the corresponding Col-0 controls that overlapped with DMRs found between the fpa complemented line and its Col-0 control.

For DRS analyses, we retrieved the data corresponding to the study PRJEB39933 deposited at the European Nucleotide Archive (Due et al., 2013). Raw DRS reads were aligned using TopHat2 (Kim et al., 2013), allowing a maximum of two mismatches and no gaps.

For transposon expression analyses, we used ibm2 and appil1 RNA-seq data described previously (Stroud et al., 2012; Saže et al., 2013; Wang et al., 2013; Duan et al., 2017). Reads were trimmed with Trim_Galore (Babraham Bioinformatics) and aligned to the Col-0 Arabidopsis TAIR10 reference genome with HISAT2 version 2.1.0 (Kim et al., 2015) using standard options. Differential expression analyses were done with the software DESeq2 (version 1.20.0; Love et al., 2014) in R (version 3.5.1). To define transposon transcripts differently expressed, we used a significance cut-off of 0.05 and a 2-fold change relative to wild type. RNA-seq read coverage files were produced and normalized with deepTools2 (Ramirez et al., 2016).
Supplemental Figure S5. Schematic representation of the IBM2 target genes and localization of the regions amplified by RT-qPCR.

Supplemental Figure S6. Characterization of plants overexpressing FPA or IBM1.

Supplemental Figure S7. Expression of IBM2 nonintronic target transposons.

Supplemental Figure S8. Host responses and Hpa EMW1A growth in Arabidopsis mutant lines.

Supplemental Figure S9. Methylation patterns of the IBM1 intron in ibm2 and fpa mutants.

Supplemental Figure S10. Methylation of IBM1 intron in fpa determined by whole-genome sequencing after bisulfite conversion.

Supplemental Figure S11. Methylation profiles of transposons derepressed in fpa.

Supplemental Table S1. Bisulfite sequencing statistics.

Supplemental Table S2. List of transposones differentially expressed in ibm2, edm2, and aip1.

Supplemental Table S3. DMRs identified between fpa-3 and the wild type in CG, CHG, and CHH contexts.

Supplemental Table S4. List of primers.

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LITERATURE CITED


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