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Report

Rapid Establishment of Genetic Incompatibility through Natural Epigenetic Variation

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Summary

Epigenetic variation is currently being investigated with the aim of deciphering its importance in both adaptation and evolution [1]. In plants, epimutations can underlie heritable phenotypic diversity [2–4], and epigenetic mechanisms might contribute to reproductive barriers between [5] or within species [6]. The extent of epigenetic variation begins to be appreciated in *Arabidopsis* [7], but the origin of natural epialleles and their impact in the wild remain largely unknown. Here we show that a genetic incompatibility among *Arabidopsis thaliana* strains is related to the epigenetic control of a pair of duplicate genes involved in fitness: a transposition event results in a rearranged paralogous structure that causes DNA methylation and transcriptional silencing of the other copy. We further show that this natural, strain-specific epiallele is stable over numerous generations even after removal of the duplicated, rearranged gene copy through crosses. Finally, we provide evidence that the rearranged gene copy triggers de novo DNA methylation and silencing of the unlinked native gene by RNA-directed DNA methylation. Our findings suggest an important role of naturally occurring epialleles originating from structural variation in rapidly establishing genetic incompatibilities following gene duplication events.

Results and Discussion

Linkage disequilibrium analysis within an *Arabidopsis thaliana* recombinant inbred line (RIL) population originating from a cross between the strains (accessions) Shahdara (Sha) and Columbia-0 (Col-0) revealed that a locus at ~13 Mb on chromosome 4 (K4) and a locus at ~26 Mb on chromosome 5 (K5) do not segregate independently [8]. Indeed, one of the four homozygous allelic combinations, namely Col-0 at the K4 locus and Sha at the K5 locus, referred to as "incompatible," is rare among the RILs: despite the strong positive selection applied on each line across generations when establishing the RIL population, we could only save 3% of these lines instead of the expected 25% [8]. This incompatible combination leads to plants with much reduced amounts of viable pollen and few seeds: under the most favorable conditions, this results in at least 80%–90% loss in seed production in comparison to other RILs of this population, which is likely to be a major defect under more selective field conditions. This pair of interacting loci is likely also involved in an incompatibility reported in lines derived from crosses between Col-0 and the accession C24 [9], because it maps to the same two genomic locations and the genotypic combination of Col-0 at the K4 locus and C24 at the K5 locus leads to plants with reduced fertility.

Fine mapping of the causative K4 and K5 loci was performed using recombinant plants in the progeny of the RIL 13RV125, heterozygous at the K4 locus and Sha at the K5 locus and of the RIL 13RV184, Col-0 at the K4 and heterozygous at the K5 locus. The K4 and K5 loci were thus narrowed respectively to 26 and 12 kb intervals that contain seven and six annotated putative open reading frames (ORFs) in Col-0. PCR amplification of each of these 13 genes revealed the existence of an extra copy of the gene *AtFOLT1* (At5g66380) in Shahdara, which we named *AtFOLT2* (Figure 1A). Using the RILs, this *AtFOLT* paralog could be mapped in Shahdara at the K4 locus involved in the incompatibility, where no homologous gene exists in Col-0. Investigating the expression of these genes, we found that *AtFOLT1* is expressed in Col-0, but not in Shahdara, whereas *AtFOLT2*, absent in Col-0, is expressed in Shahdara. The expression patterns of individual RILs agree with their genotypes: RILs with a Sha allele at the K4 locus and a Col-0 allele at the K5 locus express both *AtFOLT1* and *AtFOLT2*, whereas no expression could be detected in RILs with the reverse incompatible allelic combination (Figure 1B; see also Figure S1A available online). Moreover, the duplication and expression patterns of *AtFOLT* genes in C24 resemble those of Shahdara. We thus hypothesize that the *AtFOLT* genes are involved in both the Col-0/Shahdara and Col-0/C24 genetic incompatibilities and that the lack of *AtFOLT* transcripts in incompatible plants is responsible for the reduced fertility (Figure S1A). The first characterized allelic incompatibility relies on a comparable model of divergent evolution occurring among paralogs of an essential duplicate gene [10].

To confirm the involvement of the *AtFOLT* genes, we analyzed *folt1* homozygous insertion mutants in the Col-0 background [11]. Indeed, the Salk_059769 mutant presents the same phenotype as our incompatible lines (flowers with rare pollen and nearly empty siliques). Previous work showed that *AtFOLT1* encodes a folate transporter [12] but did not describe any phenotype for *folt1* mutants using another mutant line, Salk_005280, which we confirm shows no particular phenotype, likely because the insertion is located at the very end of the gene. A complementation test showed that among 164 plants from a cross between the Salk_059769 mutant (allele $AtFOLT1^{mut}$) and a line Col-0 at the K4 locus and heterozygous (Sha/Col) at *AtFOLT1*, 51% had an $AtFOLT1^{col}/AtFOLT1^{mut}$ genotype and were fertile, and 49% had an $AtFOLT1^{sha}/AtFOLT1^{mut}$ genotype (i.e., no functional allele of *AtFOLT*) and showed reduced fertility, confirming that the incompatibility is due to the lack of *AtFOLT* transcripts (Figure S1A). In all organisms, folates are involved in key metabolic pathways, such as amino acid and nucleotide biosynthesis. Therefore, by impacting cells with high mitotic activity, they are thought to affect initial events of reproduction from gametogenesis to early embryonic development, and indeed, folate deficiency causes reduced fertility in rats [13].

We investigated the link between the expression of *AtFOLT1* and the incompatibility in 24 additional segregating populations (F2 or RIL sets) generated from crosses between Col-0

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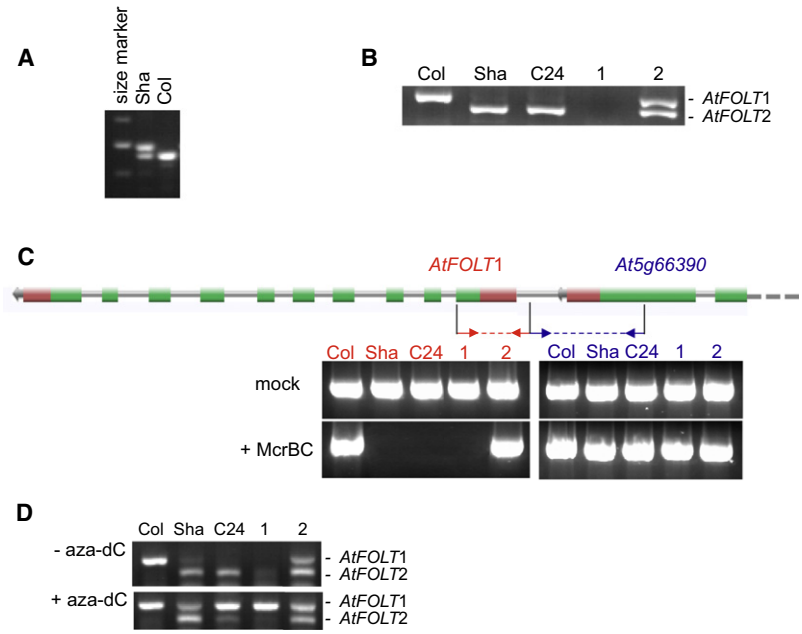

Figure 1. Amplification, Expression, and DNA Methylation of the *AtFOLT* Genes

(A) PCR amplification with primers located on the exons 2 and 4 of *AtFOLT1* shows that a second fragment of different size is present in Shahdara. This fragment corresponds to a paralog of the gene *AtFOLT1*, named *AtFOLT2*.

(B) Expression analyses of *AtFOLT1* and *AtFOLT2* in Col-0, Sha, C24, RIL 13RV5 with the incompatible combination Col K4/Sha K5 (1), and RIL 13RV2 with the compatible combination Sha K4/Col K5 (2). cDNAs were amplified with primers common to *AtFOLT1* and *AtFOLT2* and digested by SsiI, which cleaves the *AtFOLT2* but not the *AtFOLT1* amplicon. See also Figure S1A.

(C) PCR amplification of the first part of *AtFOLT1* (in red) and the end of the next gene *At5g66390* (in blue) after DNA digestion with the methylation-sensitive enzyme McrBC or a mock treatment. Genotypes are as in (B).

(D) Expression analyses of *AtFOLT1* and *AtFOLT2* in plantlets grown on medium with (+ aza-dC) or without (− aza-dC) 5-aza-2′deoxycytidine.

and different accessions (Table 1). As long as *AtFOLT1* was expressed (19 accessions), the K4 and K5 loci segregated independently in these populations and we observed no incompatible phenotype. Conversely, incompatible plants were found in the progenies of crosses involving each of the five accessions in which *AtFOLT1* was not expressed (Table 1), and these plants had the Col-0 allele at the K4 locus and the other parental allele at the K5 locus.

Table 1. Incompatibility and *AtFOLT1* Expression in 24 Segregating Populations

Accession	Geographical Origin	Segregating Population (× Col-0)	AtFOLT Incompatibility	*AtFOLT1* Expression
Bla-1	Spain	RIL	−	+
Blh-1	Czech Republic	RIL	−	+
Bur-0	Ireland	RIL	−	+
Can-0	Canary Islands	RIL	−	+
Ct-1	Italy	RIL	−	+
Cvi-0	Cape Verde Islands	RIL	−	+
Dja-1	Kyrgyzstan	F2	−	+
Ge-0	Switzerland	RIL	−	+
Ishikawa	Japan	F2	−	+
Ita-0	Morocco	RIL	−	+
Jea	France	RIL	−	+
Kar-1	Kyrgyzstan	F2	+	−
Kyr-1	Kyrgyzstan	F2	+	−
Ler	Poland	F2	−	+
Ms-0	Russia	F2	+	−
Nok-1	Netherlands	RIL	−	+
Oy-0	Norway	RIL	−	+
Ri-0	Canada	RIL	−	+
Rld-2	Russia	F2	+	−
Sakata	Japan	F2	−	+
Sus-1	Kyrgyzstan	F2	−	+
Tsu-0	Japan	RIL	−	+
Yo-0	USA	RIL	−	+
Zal-1	Kyrgyzstan	F2	+	−

In order to determine the cause of the nonexpression of *AtFOLT1* in Shahdara, we sequenced the 12 kb fine-mapped K5 interval in Shahdara and several other accessions. A total of 29 SNPs were found between Shahdara and Col-0. However, in the accession Ishikawa, where *AtFOLT1* is expressed, this sequence is perfectly identical to that of Shahdara, suggesting that the absence of *AtFOLT1* transcript in Shahdara could result from epigenetic silencing. In support of this, we show that the promoter and the first part of *AtFOLT1* are methylated in Shahdara, C24, and the RILs where *AtFOLT1* is not expressed, whereas, as in Col-0 ([14]; http://neomorph.salk.edu/epigenome/epigenome.html), they are unmethylated in lines where *AtFOLT1* is expressed (Figure 1C). This methylation is limited to *AtFOLT1* and does not affect upstream sequences (Figure 1C). To confirm that DNA methylation is associated with *AtFOLT1* silencing, plantlets were grown on medium containing 5-aza-2′deoxycytidine, which inhibits cytosine methylation: this treatment induced the expression of *AtFOLT1* in plantlets descending from silenced plants (Figure 1D). Furthermore, sequencing after bisulfite treatment of genomic DNAs indicates that *AtFOLT1* DNA methylation is not restricted to CG sites but also affects CHG and CHH sites (Figure 2), a hallmark of RNA-directed DNA methylation (RdDM) [15]. Consistent with this, 21 and 24 nt small RNAs matching *AtFOLT* sequences are detected in Shahdara and C24, but not in plants devoid of *AtFOLT2* sequences such as Col-0 and incompatible lines (Figure 3). These small RNAs could thus originate from the *AtFOLT2* locus and initiate the methylation of homologous sequences (Figure S1B). Regions containing repeated sequences can trigger the biogenesis of small interfering RNAs (siRNAs) targeting in *trans* the DNA methylation of homologous sequences through the RdDM pathway [15].

Molecular characterization of the *AtFOLT2* locus revealed a complex organization, with *FOLT*-like sequences interspersed with duplicated sequences of K4, sequences with no homologies in the Col-0 genome and even sequences homologous to the chloroplastic genome. Actually, this locus is so complex that even interrogating recent genome

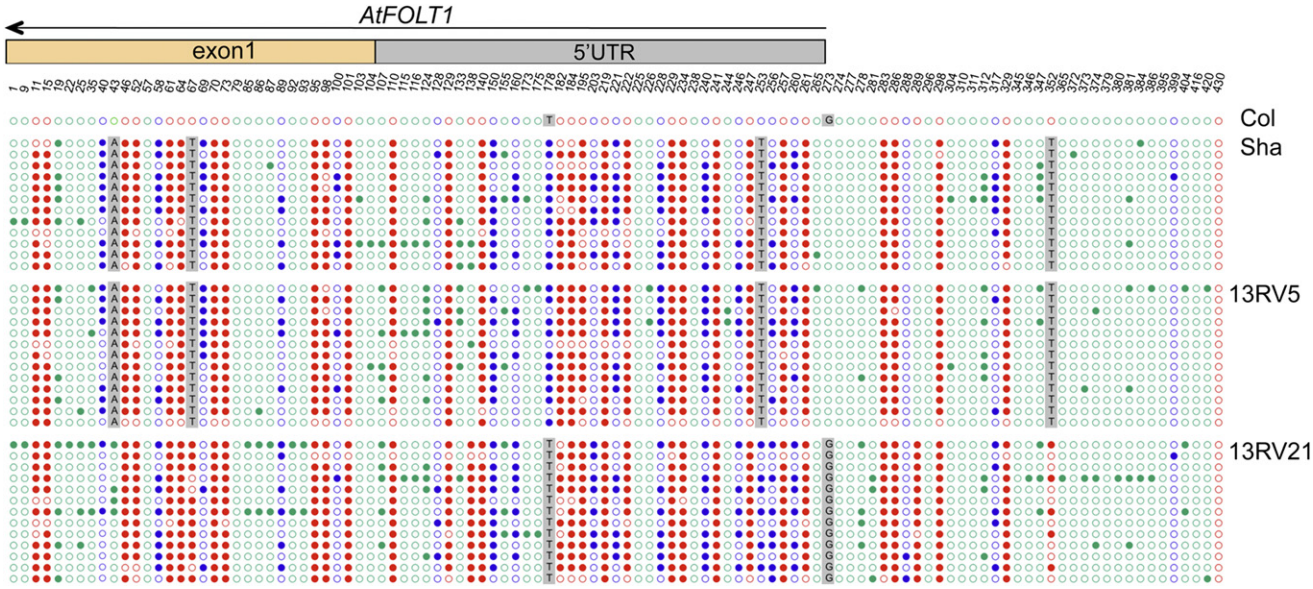

Figure 2. Analysis of *AtFOLT1* DNA Methylation by Bisulfite-Treated DNA Sequencing

The sequenced region corresponds to the region between red arrows in Figure 1C. Cytosines are represented by circles (red, CG; blue, CHG; green, CHH; solid circles, methylated cytosines), and their position within the sequence (chr5: 26,515,428-26,515,857 -TAIR10) is indicated. Sequence polymorphisms between Col-0 and Shahdara are in gray. Only one Col-0 sequence is shown because all the sequences were identical with no cytosine methylation. The Col-0 sequences also served for checking the completeness of the bisulfite conversion. In the region upstream of *AtFOLT1*, the first 300 bp following the stop codon of At5g66390 are CG depleted (four CG and three CHG occurrences in Sha) and totally unmethylated. In contrast, the next region, which encompasses the beginning of *AtFOLT1* until the end of exon 1, is CG rich (29 CG and 17 CHG occurrences within 340 bp in Sha). In this region, all of the CG are highly methylated both in Shahdara (at 90% on average) and in the incompatible line 13RV5 (at 89% on average), and all of the CHG are partially methylated (at 43% on average in Shahdara and 40% in 13RV5). The pattern of de novo methylation in 13RV21 in generation F7 (compatible line in which *AtFOLT1* is not expressed any more) is identical to those of Shahdara and 13RV5; additional CG and CHG that are present in this region in the Col allele compared to the Sha one are also methylated.

sequencing data ([16] and http://1001genomes.org/projects/assemblies.html) has not allowed us to determine its entire sequence. However, we could show that the K4 locus in Shahdara comprises two additional rearranged truncated sequences homologous to parts of *AtFOLT2*, one of them and the complete *AtFOLT2* being associated with duplicated sequences from chromosome 4 (Figure 4). These specific fragments do not exist in Col-0, and they were mapped on the Shahdara genome by using the Col × Sha RIL population and recombinant plants that served to fine map the chromosome 4 locus. PCR amplification, after DNA digestion with the methylation-sensitive enzyme McrBC, of the regions of these *AtFOLT*-like sequences that are homologous to the methylated part of *AtFOLT1*, showed that all these regions are methylated (Figure 4). This suggests that the factors triggering the methylation, likely the siRNAs, do act in *trans* on all homologous sequences. Although no transcription is expected from methylated promoters, two polyadenylated RNAs corresponding to the complete copy and one rearranged truncated copy of *AtFOLT2* were detected in Shahdara. However, both are chimeric mRNAs comprising part of the 5′ untranslated region (5′UTR) of At4g27050 (Figure 4), therefore initiated from the unmethylated promoter of this upstream gene (Figure S1B). Bisulfite sequencing data across this region show that the methylation is restricted to the *AtFOLT* sequence and does not affect the heterologous promoter (Figure S2). The aberrant RNA originating from the rearranged truncated *AtFOLT2* copy, which contains *AtFOLT* promoter sequences, could give rise to the siRNAs that control the methylation of homologous promoter sequences and the silencing of the target gene *AtFOLT1*. A comparable example was reported for the *PAI* gene family in *Arabidopsis*, where a *PAI* inverted repeat is associated with the presence of siRNAs that trigger the methylation and silencing of homologous genes in *trans* [17–19].

The phenotype of incompatible lines is extremely stable across generations. We screened several dozens of plants in successive generations of the different incompatible lines

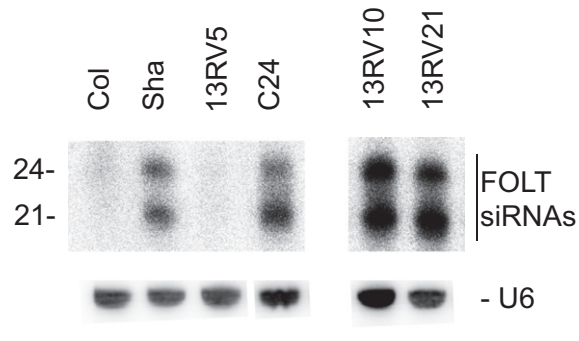

Figure 3. *AtFOLT* Small RNAs

Northern blot analyses with a riboprobe corresponding to the promoter and the first exon of *AtFOLT1* detect small RNAs of 21 and 24 nt homologous to the methylated part of *AtFOLT1* in different genotypes. 13RV5, RIL with the incompatible combination Col K4/Sha K5; 13RV10 and 13RV21, RILs with the compatible combination Sha K4/Col K5. U6 RNA hybridization was used as a loading control. See also Figures S1B and S3.

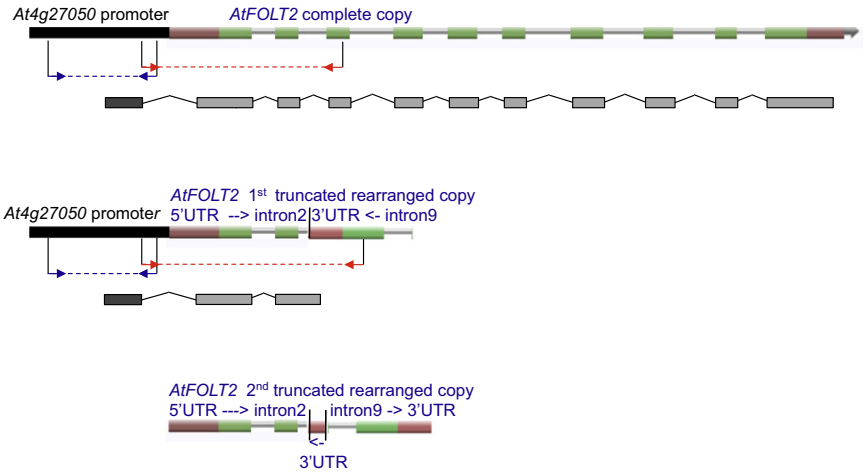

Figure 4. The Shahdara *AtFOLT2* Locus Comprises Repeated *AtFOLT* Sequences

Three *AtFOLT*-like sequences were amplified by PCR and mapped at this locus. The first one (2,365 bp) comprises the complete copy of *AtFOLT2*, the *AtFOLT1* paralog, composed of ten exons (green bars) and nine introns. The red bars represent genomic regions homologous to the 5′ and 3′ sequences of *AtFOLT1*. The two other *AtFOLT*-like copies (1,102 and 627 bp) are rearranged truncated *AtFOLT2* sequences; both comprise the beginning of *AtFOLT2*, from the 5′UTR until intron 2, associated with different sequences from the end of the gene, some of them in reverse orientation (as indicated by arrows). The complete *AtFOLT2* copy and one rearranged copy are associated with the same duplicated sequence from K4 (black bar), which includes the promoter of the At4g27050 gene. The PCR fragments amplified in the McrBC test are indicated; after McrBC digestion, the fragments encompassing the region that is methylated in *AtFOLT1* (in red) are not amplified and therefore include methylated sequences, and K4 regions including the *At4g27050* promoter (in blue) are amplified and unmethylated. Bisulfite sequencing data across this region are shown on Figure S2. The chimeric cDNAs corresponding to the complete copy and the first rearranged truncated copy of *AtFOLT2* are shown (gray bars); both start with part of the 5′UTR of At4g27050 (dark gray).

and never saw a reversion. In addition, we have followed the methylation and expression status of the *AtFOLT1* gene in successive generations of these lines and found *AtFOLT1* to be always methylated and silenced, even when the inducing locus was segregated away six generations ago. The siRNAs, which are not produced in these lines (Figure 3), are thus not necessary to maintain the methylation and silencing of *AtFOLT1*, at least for a few generations. Because in Shahdara and in incompatible RILs most of the methylated cytosines are in symmetrical contexts (CG and CHG), the methylation is certainly maintained by the methyltransferases MET1 and CMT3 [20]. In plants, other genes epigenetically silenced by DNA methylation are also stably transmitted over several generations [21].

Next, we investigated more precisely *AtFOLT1* expression in 15 RILs harboring the compatible genotypic combination Sha at the K4 and Col-0 at the K5 loci (Figure S1A). In most of these lines, *AtFOLT1*, originating from Col-0, is expressed. However, we found that in one of these lines (13RV21), although *AtFOLT1* is unmethylated and expressed at the F3 generation, it is methylated and not expressed anymore at the F7 generation (Figure S3). Some other lines with the same genotype, such as 13RV10, show a reduced expression of *AtFOLT1* at the F7 generation (Figure S3), followed by a complete silencing of the gene in subsequent generations. Sequencing of *AtFOLT1* in 13RV21(F7) after bisulfite treatment shows a methylation pattern identical to that of the Shahdara *AtFOLT1* gene (Figure 2). The Shahdara *AtFOLT2* locus is thus able to *trans*-induce de novo methylation of the originally unmethylated $AtFOLT1^{col}$ sequence. This methylation is set up progressively and stochastically through generations, as previously observed for newly introduced transgenes [22] or certain siRNA-targeted transposons that have been artificially demethylated [23]. This is consistent with a siRNA-mediated RdDM mechanism targeting the methylation of *AtFOLT1*, and this was confirmed by the detection of *AtFOLT* siRNAs in both 13RV21 and 13RV10 (Figure 3).

A small number of genes with natural methylation variation leading to different phenotypes have been described in plants, which change floral symmetry in *Linaria vulgaris* [2], fruit ripening in tomato [3], sex determination in melon [4], or plant stature in rice [24]. In maize, the *b1* locus, which affects plant pigmentation, is a classic case of paramutation, a transfer of epigenetic information between two alleles of a gene resulting in heritable gene expression changes [25]. It is thought that paramutation could play a role in adaptation and evolution by conferring favorable expression states to the next generations [26]. In *Arabidopsis*, recent studies have described spontaneous transgenerational epigenetic changes in DNA methylation [27] and suggested that the new epialleles may alter transcription and contribute to phenotypic variation [28]. However, only a few examples of loci producing siRNAs and associated DNA methylation have been described specifically in certain *Arabidopsis* accessions [17, 19, 29, 30]. The PAI and AtFOLT systems rely on similar silencing mechanisms, but, because the accessions in which the *PAI* genes are not silenced contain several functional copies of the gene, crosses between accessions do not result in incompatible allelic combinations. The *AtFOLT* genes thus represent the first reported case of a natural epiallele that has strong deleterious phenotypic consequences steadily maintained in the progenies of crosses between accessions. Because the newly rearranged structure is able to trigger in *trans* the silencing of the distant original copy of the gene in just a few generations and at high frequency, this gene transposition event can promptly set up an allelic incompatibility among accessions, and the rapid establishment of the epigenetic control may be an important factor in accelerating evolution [31]. Moreover, the silencing mechanism would be efficient even while the rearranged duplicate is in a heterozygous state. An additional contrast to the previously characterized recessive incompatibility [10] is that silencing by epigenetic mechanisms does not require protein loss of function and is potentially reversible. This could help maintain the capacity to evolve to sub- or neofunctionalization among duplicates [32]. This kind of naturally occurring epiallele affecting important genes involved in plant fitness could thus, alone or combined together, rapidly impact plant populations and constitute an underlying factor in evolution.

Experimental Procedures

Plant Material

Shahdara × Col-0 RILs (named 13RV), as well as other RIL and F2 sets, are described at and obtained from http://dbsgap.versailles.inra.fr/vnat/ (Institut Jean-Pierre Bourgin Versailles). Fine mapping was performed by genotyping recombinant plants in the progenies of RILs 13RV125 and 13RV184 with microsatellite markers. For 5-aza-2′deoxycytidine treatment, sterilized seeds were sown in vitro on Murashige and Skoog medium containing 2 mg/l 5-aza-2′deoxycytidine, and plantlets were harvested after 2 weeks to undergo RNA isolation.

Analysis of DNA Methylation by Bisulfite Sequencing

Bisulfite treatment was performed on 1 μg of genomic DNA using the Epitect Bisulfite kit (QIAGEN). The following modified PCR primers were designed with Methyl Primer Express software (Applied Biosystems): *AtFOLT1*, 5′-CTATATACTATTTACTTTATTTCC-3′ and 5′-GATGAGTTAATTTAGGAG TGAA-3′; *AtFOLT2*, 5′-TTTATATCAATCTCTTACTCTCA-3′ and 5′-TTTAG TTAGTTGTAGGAGTGAA-3′. Amplified fragments were cloned before sequencing and data analyzed by Kismeth software [33]. At least 12 clones of each genotype were sequenced.

PCR Analysis of the *AtFOLT* Paralogs

Primers used to differentiate *AtFOLT1* and *AtFOLT2* based on the size of the amplicon (Figure 1A) were 5′-TCAGCAGCAGAAGCAAGGT-3′ and 5′-AACGACGGAAGAGGGTCAAG-3′. *AtFOLT1* and *AtFOLT2* cDNAs were both amplified with common primers 5′-CTGAAGAAGACACGGTCGAC-3′ and 5′-AGGGGCCTTGGTCTGTTTAT-3′ in a 396 bp fragment. SsiI specifically cleaves the *AtFOLT2* amplicon into 309 and 87 bp fragments.

The following primers were used for McrBC digestion assays: *AtFOLT1*, 5′-TCCAGGAGTGAAAAACCAGA-3′ and 5′-ATTTCCAGCGTTGAACTTGC-3′; At5g66380, 5′-GCAAGTTCAACGCTGGAAA-3′ and 5′-GAGCAAGCTTGAG TGGTTCC-3′; *AtFOLT2* sequences, 5′-CCTTAAGCTTTTTACTCTGGT TC-3′ and 5′-AAAGAAGTATAAGCCCCAGGA-3′ (complete copy) or 5′-CCA TCACATTCATCGTCTATG-3′ (rearranged copy); At4g27050, 5′-AAGTT GTCGTGTTGCTTTGAG-3′ and 5′-GGATGTCGATGATGAAGAGGA-3′.

Small RNA Analyses

Small RNAs (10 μg) were extracted from flowers and separated as described [34]. A radiolabeled RNA probe transcribed in vitro (from an amplicon obtained with primers 5′-GATGCATTAACCCTCACTAAAGGGACTTGAA CGGACGGCAGAAC-3′ and 5′-GATGCTAATACGACTCACTATAGGGAGA GAGTGCATAGCAGCTAC-3′) was used to detect *AtFOLT* siRNAs.

Supplemental Information

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.cub.2011.12.054.

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