Transposons and Tandem Repeats Are Not Involved in the Control of Genomic Imprinting at the *MEDEA* Locus in *Arabidopsis*

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Transposons are selfish mobile DNA elements that can insert into nonhomologous target sites, thereby amplifying their copy number in the genome. Yet, transposition is considered tightly controlled, because unregulated amplification could have severe consequences for the fitness of the host organism. Nonetheless, transposons constitute ~45% of the human genome and ~80% of the maize genome. Transposable elements can act as both "attractors" and "mediators" of epigenetic regulation across the genome. The potential for transposons to show epigenetic activity leading to effects on phenotypic variation was first recognized by McClintock (see McClintock 1984) as "changes in phase." Some years ago it was proposed that transposons may be involved in a variety of epigenetic phenomena such as gene silencing, paramutation, and genomic imprinting (Martienssen 1996; Matzke et al. 1996). Indeed, there is growing evidence that transposons can act as epigenetic mediators of phenotypic variation. Here, we briefly review the role of transposons and repeated sequences in epigenetic gene regulation and investigate their potential role in controlling genomic imprinting at the *MEDEA* locus of *Arabidopsis*.

TRANSPOSONS AND REPETITIVE SEQUENCES AS EPIGENETIC ATTRACTORS AND MEDIATORS

Transposons are well known to be evolutionary drivers of chromosomal repatterning by reorganizing genome structure through transposition and by causing chromosomal rearrangements such as deletions, inversions, and translocations. But they can also modulate the transcription patterns of genes adjacent to the sites of transposon insertions (for review, see Kazazian 2004). Over evolutionary time most transposons have accumulated mutations that render them incapable of transposition, but many of their promoters remain active (Whitelaw and Martin 2001). Retrotransposons often have strong constitutive promoters that can affect the transcription of adjacent genes. Indeed, transposons can serve as alternative promoters for many mammalian genes (van de Lagemaat et al. 2003). For instance, transposon insertions proximal to genes can lead to overexpression causing hypermorphic alleles or to the production of chimeric transcripts that encode proteins with anti- or neomorphic activity. Such effects have been demonstrated for the *agouti* (Michaud et al. 1994; Argeson et al. 1996) and the mouse intracysternal A-type particle (IAP)-promoted *Mipp* gene (Chang-Yeh et al. 1993).

The effects described above may be viewed as a consequence of genetic changes since they rely on a read-through transcription from a transposon promoter and thus a restructured genome. However, transposons may also provide a link between genetic and epigenetic processes, via their activities as both transcribed genes and *cis*-acting repeats. In many organisms, duplicated or repetitive elements including transposons act as epigenetic "attractors" of mechanisms that lead to their inactivation or reduce their copy number (Matzke et al. 1996; Yoder et al. 1997; Whitelaw and Martin 2001; Lyon 2003). The mechanisms differ between repeat systems and organisms, but involve many of the classical epigenetic regulatory systems, such as DNA methylation, chromatin modification, and transcriptional interference.

DNA Methylation and Histone Modifications Regulate Transposon Activity

It has been proposed that cytosine DNA methylation acts primarily to suppress transcription from "intragenomic" parasitic elements (e.g., transposons) across the genomes of higher eukaryotes (Matzke et al. 1996; Yoder et al. 1997; Bestor 2003). DNA methylation can suppress transposition by making the transposon inaccessible to its transposase. In addition, transposon promoters can be inactivated by methylation either epigenetically or genetically, because of the increased frequency of C $\rightarrow$ T base transitions at methylated sites. Studies in both mammals and plants have demonstrated that demethylation of the genome can trigger remobilization of epigenetically silenced transposons (Walsh et al. 1998; Hirochika et al. 2000; Kato et al. 2003). It is likely that the majority of cytosine methylation found in eukaryotic genomes is associated with suppression of multicopy transposons and centromeric satellite DNA (both enriched for CpG content). This seems to be the case for plants and fungi where methylation is mainly associated with transposons and repetitive DNA, whereas in mammals coding regions also

can be methylated (Martienssen and Colot 2001; Lippman et al. 2004). Transposon insertions may also affect adjacent genes via spreading of CpG methylation into "innocent bystander" genes, leading to their silencing. Yates and colleagues (1999) demonstrated such an effect for tandem B1 repetitive elements on the silencing of the adjacent adenine phosphoribosyltransferase *Aprt g*ene in mice.

In recent years it has become clear that histone modifications play an important role in the epigenetic regulation of gene activity (for review, see Imhof 2003; Wang et al. 2004). There are mechanistic links between DNA methylation and histone modifications affecting chromatin structure (for review, see Tariq and Paszkowski 2004), and transposons can also be suppressed by targeting them for heterochromatin formation, which would act to suppress their transcription, mobility, and recombinational activity. Indeed, studies of transposons have demonstrated that transposon loci are subject to histone methylation (Rea et al. 2000; Gendrel et al. 2002). For instance, *Arabidopsis* mutants affecting epigenetic regulation were investigated for effects on the activity and inheritance of six transposon classes (Lippman et al. 2003). It was found that two distinct epigenetic mechanisms silence transposons and that transposon silencing complexes interact via histone modifications and RNA interference (RNAi). There is mounting evidence for a role for RNAi in chromatin modifications that regulate transposable element activity at centromeric heterochromatin (Volpe et al. 2002; Dawe 2003). In *Caenorhabditis elegans,* RNAi-deficient strains exhibit mobilization of endogenous transposons indicating that the RNAi machinery is involved in suppression of transposon activity (Tabara et al. 1999). The emerging picture is that heterochromatic regions can generate small RNAs that direct an RNAi-based modification of the chromatin in heterochromatic repeats and transposable elements (Bender 2004; Lippman and Martienssen 2004).

Transposons and Classical Epigenetic Phenomena

Metastable epialleles are alleles where the epigenetic state can switch and be mitotically inherited, yet the establishment of the epigenetic state is a probabilistic event (Rakyan et al. 2002). All metastable alleles that have been investigated at the molecular level have been shown to be associated with a transposon insertion (Rakyan et al. 2002). For instance, Rakyan and coworkers (2003) have demonstrated a role for retrotransposon-based regulation of the classical metastable mutant allele Axin-fused ($Axin^{Fu}$). The presence or absence of the $Axin^{Fu}$ phenotype, a kinked tail, correlated with differential DNA methylation of a retrotransposon within $Axin^{Fu}$. Affected transcripts arising adjacent to the retrotransposon long terminal repeat (LTR), usually containing a promoter, are considered as likely causes of the phenotype. A similar case was described for an *agouti* allele, where the insertion of an IAP retrotransposon into the upstream region caused a range of phenotypes that showed partial epigenetic maternal inheritance due to incomplete erasure of the epigenetic modification at *agouti* (Morgan et al. 1999).

Transposons are also proposed to play a role in mammalian X-chromosome inactivation. Interspersed repeats, in particular long interspersed nucleotide elements (LINEs), have been suggested as features that act as attractors of the X-inactivation machinery (Lyon 2003). In support of this model is the observation that L1 LINE content is lower in regions of the X chromosome containing genes that escape inactivation (Bailey et al. 2000).

Several years ago it was proposed that transposons and repetitive elements may be mechanistically linked to the phenomenon of paramutation (Martienssen 1996; Matzke et al. 1996; Della Vedova and Cone 2004). Paramutation is an allelic interaction that results in meiotically heritable changes in gene expression (Brink 1973). By analyzing the physical structure of 28 haplotypes at the *red1* (*r1*) locus of maize, a strict correlation of paramutability (the ability to become silenced) and structural features could be established (Walker and Panavas 2001). The *r1* locus is complex, often containing several *r1* gene copies encoding helix-loop-helix transcription factors. All paramutable alleles contain an *S* subcomplex that includes two *S* genes (*r1* homologs) forming a head-to-head inverted repeat and a *q* gene fragment (homologous to the *r1* promoter). These elements of the *S* subcomplex usually contain sequences derived from a *Doppia* transposable element. The paramutagenicity (the ability to cause silencing) of *r1* haplotypes, on the other hand, does not correlate with structural features but paramutagenic alleles show consistently higher levels of DNA methylation (Walker and Panavas 2001). At the *booster1* (*b1*) locus, which is also subject to paramutation, Stam and coworkers (2002) have shown that tandem repeats of an 853-bp sequence located ~100 kb upstream of the *b1* gene are required for paramutagenicity. A further link between paramutation and transposons is illustrated by the fact that paramutation at three different loci in maize and silencing of *Mutator* transposable elements are coordinately affected in certain inbred backgrounds (Walbot 2001) and by mutations at the *modifier of paramutation 1* (*mop1*) locus (Dorweiler et al. 2000; Lisch et al. 2002).

EPIGENETIC REGULATION OF TRANSPOSONS AND IMPRINTED GENES

Genomic imprinting refers to an epigenetic phenomenon where paternally and maternally inherited alleles are expressed differentially after fertilization. Most imprinted genes in mammals display parent-of-origin-specific methylation patterns. Compelling evidence that transposons are not neutral genomic parasites but actively influence epigenetic gene regulation poses the question whether they play a role in genomic imprinting as well. Indeed, the epigenetic regulation of some transposons is analogous to that of imprinted genes whereby an autosomal locus can be differentially expressed depending on the sex of the parent from which it was inherited. In mammals, L1 elements and IAP retroviruses are methylated when inherited paternally, but not methylated when inherited maternally (Sanford et al. 1987). The opposite situation occurs for mammalian Alu elements (Rubin et al.

1994). Early during mouse embryogenesis, genome-wide DNA demethylation occurs, followed by de novo remethylation. For most imprinted genes, the unmethylated allele escapes postimplantation de novo methylation; differentially methylated transposons, however, do not (Yoder et al. 1997; Walsh et al. 1998).

A survey of more than 30 imprinted genes brought the first correlative evidence for a possible involvement of transposons in genomic imprinting. Neumann et al. (1995) highlighted that one of the characteristics of known imprinted genes was that they tended to be enriched in short direct repeats. In mammals, the accumulation of short interspersed nucleotide elements (SINEs) is constrained in promoter regions of imprinted genes, whereas L1 LINE transposons preferentially accumulate in the vicinity of paternally expressed imprinted genes (Greally 2002; Fazzari and Greally 2004). Furthermore, this dual feature of imprinted regions points toward a mechanistic role of the transposons, where paucity in one type (the SINEs) would ensure that imprinted regions are isolated in a distinct genomic compartment, potentially enabling distinct regulatory mechanisms, and the other type (the L1 LINEs), being asymmetrically distributed, would provide a genomic signature to undergo preferential maternal or paternal silencing in the gametes. This remains a postulate, which needs to be experimentally verified, but it opens the field of investigation toward elucidating the mechanisms of genomic imprinting.

GENOMIC IMPRINTING IN PLANTS

Long before genomic imprinting was studied in mammals, Kermicle (1970) demonstrated that specific alleles of the maize *r1* locus are regulated by genomic imprinting (for review, see Kermicle 1994; Baroux et al. 2002). Maternal and paternal alleles of several other maize and, more recently, *Arabidopsis* genes were shown to be differentially expressed during seed development. However, of all plant genes suggested to be regulated by genomic imprinting, the maternally inherited allele is active, and most of these genes are already expressed prior to fertilization. Thus, although unlikely, the differential steady-state levels of maternally and paternally derived transcripts might be due to expression of the maternal allele prior to fertilization and not due to active expression postfertilization. In such cases, a clear demonstration of genomic imprinting requires not only the detection of differential expression levels of maternally and paternally derived transcripts but also an assay showing that the maternal allele is actively transcribed in at least one of the products of double fertilization (embryo and endosperm).

Regulation by genomic imprinting was proposed for several maize genes that are active in the endosperm, including specific alleles of *r1* (Kermicle 1970), the *delta zein regulator1* gene (*dzr1*; Chaudhuri and Messing 1994), and specific α-tubulin and *zein* genes (Lund et al. 1995a,b). Except for *dzr1*, which is not yet cloned, the high expression levels of maternal genes correlated with hypomethylation of the maternally inherited alleles (for review, see Alleman and Doctor 2000; Baroux et al. 2002). For *r1*, genomic imprinting could unambiguously be demonstrated in an elegant genetic analysis that excluded a prefertilization component (for instance, long-lived, stored transcripts [Kermicle 1970]). For the other potentially imprinted loci, early expression in ovules was not analyzed; therefore, a prefertilization cause for differential transcript levels cannot unambiguously be excluded. Recently, three additional endosperm-specific maize genes have been described that show differential expression levels during seed development but are not expressed at all prior to fertilization. Thus, *ZmFie1* (Springer et al. 2002; Danilevskaya et al. 2003), *no apical meristem related protein1* (*nrp1*; Guo et al. 2003), and *maternally expressed gene1* (*meg1*; Gutiérrez-Marcos et al. 2004) are clearly regulated by genomic imprinting.

The *FIS*-Class Genes in *Arabidopsis*

In *Arabidopsis*, the *MEDEA* (*MEA*) gene, which was isolated in a screen for gametophytic maternal effect mutations (Grossniklaus et al. 1998), was shown to be regulated by genomic imprinting. Differential expression levels of transcripts derived from the two parental alleles were demonstrated by allele-specific reverse transcriptase polymerase chain reaction (RT-PCR) (Kinoshita et al. 1999; Vielle-Calzada et al. 1999) and active transcription after fertilization was shown using an in situ hybridization method analogous to RNA-FISH (fluorescence in situ hybridization) (Vielle-Calzada et al. 1999). This analysis showed that only two of the three *MEA* copies present in the endosperm are actively transcribed. Although the latter method could be applied only to endosperm nuclei, allele-specific quantitative PCR analyses showed that paternally derived transcripts were not detectable at any stage of seed development (up to 10 days after pollination; Page 2004). Thus, the *MEA* gene, which is expressed in both embryo and endosperm (Vielle-Calzada et al. 1999), is likely regulated by genomic imprinting in both fertilization products.

MEA encodes a *Polycomb* group (PcG) protein with high similarity to *Enhancer of zeste* from *Drosophila* (Grossniklaus et al. 1998). Several other independent screens identified additional loci with similar parent-of-origin-dependent phenotypes (Ohad et al. 1996; Chaudhury et al. 1997; Guitton et al. 2004). This class of mutations is referred to as the *fis* class (Grossniklaus et al. 2001) and includes the *MEA* (Grossniklaus et al. 1998), *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE;* Ohad et al. 1999), *FERTILIZATION-INDEPENDENT SEED2* (*FIS2;* Luo et al. 1999), and *MSI1* (Köhler et al. 2003) genes. These *FIS* proteins form a multiprotein complex that is analogous to the E(z)-Esc complex of *Drosophila* and the Enx-Eed complex of mammals (Köhler et al. 2003; for review, see Reyes and Grossniklaus 2003). Differential expression of maternally and paternally inherited alleles has been described for the *FIE* and *FIS2* genes (Luo et al. 2000; Yadegari et al. 2000). However, since both of these genes are also expressed prior to fertilization (Luo et al. 2000: Spillane et al. 2000; Yadegari et al. 2000) and active transcription at postfertilization

stages has not been investigated, regulation by genomic imprinting has not been demonstrated unambiguously.

Transposons and Genomic Imprinting at the *FWA* Locus

Recently, genomic imprinting was reported for an additional *Arabidopsis* gene: the *FWA* locus (Kinoshita et al. 2004), which was originally identified as a late flowering mutant (Koornneef et al. 1991). Late flowering in the *fwa* epimutant is caused by ectopic expression of the *FWA* gene due to hypomethylation of repeats upstream of the transcriptional start site (Soppe et al. 2000). Kinoshita et al. (2004) found that *FWA* is expressed in the endosperm and could detect transcripts derived only from the maternally inherited allele, suggesting the gene may be regulated by genomic imprinting. However, as *FWA* is also expressed prior to fertilization and a conclusive test for active transcription at postfertilization stages has not been performed, the definitive proof is missing. Nevertheless, it is highly likely that *FWA* is regulated by genomic imprinting because it also shares upstream regulators with *MEA*. On the one hand, the maternal activity of both *MEA* and *FWA* depends on *DEMETER* (*DME*), a gene that was identified based on its phenotype that is similar to that of *mea* (Choi et al. 2002; Guitton et al. 2004). *DME* encodes a DNA-glycosylase homolog whose activity is required for an active maternal *MEA* allele (Choi et al. 2002, 2004). On the other hand, the DNA methyltransferase *MET1* is a regulator of both genes. However, while *MET1* was proposed to act antagonistically to *DME* on the maternal *MEA* allele (Xiao et al. 2003), it repressed the paternal *FWA* allele (Kinoshita et al. 2004), while *MET1* does not seem to affect the paternal *MEA* allele (Luo et al. 2000).

Interestingly, at the Symposium, Robert Martienssen (see Martienssen et al., this volume) reported a link between the imprinted control of *FWA* expression and transposons. His group investigated McClintock's hypothesis (1952) that transposons ("controlling elements") might reside in heterochromatic regions (for instance, heterochromatic knobs), but also exercise regulatory functions across the genome. In support of this hypothesis, Lippman and coworkers (2004) have demonstrated that heterochromatin in *Arabidopsis* is determined by transposons and related tandem repeats, which are epigenetically regulated by the chromatin remodeling ATPase DDM1. It was further shown that transposons can exercise epigenetic regulation of adjacent genes and that this was the likely explanation for the epigenetic inheritance patterns observed at the imprinted *FWA* gene. In addition, small interfering RNAs (siRNAs) associated with the epigenetically regulated transposon type were found in both heterochromatin and the promoter of the *FWA* locus. It was proposed that the transposon brings the *FWA* locus under the control of *DDM1* and is responsible for its epigenetic regulation (Lippman et al. 2004).

Over the last few years, we have investigated the potential role of transposable elements and repeated sequences in the regulation of the imprinted *MEA* locus. Unlike for *FWA*, no evidence for a role of repeats was found, suggesting that different mechanisms are responsible for the regulation of *MEA* and *FWA* by genomic imprinting.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

The *mea-1* mutant line in Ler-0 genetic background used was previously described (Grossniklaus et al. 1998). All *Arabidopsis* ecotypes were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. Seeds were surface sterilized using 2% sodium hypochlorite and allowed to germinate on Murashige and Skoog (MS) medium (Duchefa) supplemented with 10 g/l of sucrose, 8 g/l of agar prior to transfer of seedlings to soil. To generate interecotype F1 hybrids between the *mea-1* mutant and the ecotypes Ler-0, Yo-0, and Kb-0, each ecotype was crossed with pollen from a *mea-1* plant and the hybrid F1 progeny seeds were selected on MS medium containing 50 mg/L of kanamycin (Sigma) as described above. Interecotype F1 *mea-1* seedlings displaying kanamycin resistance were chosen for further analysis. Seedlings of both ecotypes and F1 interecotype progeny were transplanted to "ED73 mit Bims" soil (Tränkle Einheiteserde) and transferred to a growth chamber with 70% humidity and a day/night cycle of 16 hours light at 21°C and 8 hours dark at 18°C.

A set of 50 evolutionarily divergent ecotypes (comprising a core collection kindly provided by Tom Mitchell-Olds, MPI Jena) was chosen for genetic and molecular analyses. These ecotypes were (stock center accession numbers in parentheses) L*er*-0 (CS20), Mh-0 (CS904), Blh-1 (CS1030), Cit-0 (CS1080), Co-1 (CS1084), Col-0 (CS1092), Cvi-0 (CS1096), Di-0 (CS1106), Ei-2 (CS1124), Est-0 (CS1148), Fe-1 (CS1154), Ga-0 (CS1180), Gr-1 (CS1198), Gu-0 (CS1212), Ha-0 (CS1218), Ita-0 (CS1244), Kas-1 (CS1264), Kb-0 (CS1268), Kil-0 (CS1270), Kin-0 (CS1272), Le-0 (CS1308), L1-0 (CS1338), Lo-1 (CS1346), Lz-0 (CS1354), Me-0 (CS1364), Nd-0 (CS1390), Np-0 (CS1396), Nok-0 (CS1398), Pa-1 (CS1438), Pla-0 (CS1458), Pog-0 (CS1476), Rsch-0 (CS1490), Ru-0 (CS1496), Sah-0 (CS1500), Ta-0 (CS1548), Tu-1 (CS1568), Uk-1 (CS1574), Ws-0 (CS1602), Wt-1 (CS1604), Yo-0 (CS1622), WI-0 (CS1630), Wei-0 (CS3110), RLD1 (CS913), XX-0 (N1618), Mt-0 (N1380), Ko-2 (N1288), C24 (N906), CS22491, CS22495, CS22484, CS22493, and Hodja.

Molecular Biology

Genomic DNA template for PCR and sequence analysis of each of the ecotypes was extracted as described (Edwards et al. 1991). To determine whether the *MEA-AtREP2* helitron was present in each ecotype, a PCR assay was developed using two primers spanning the *MEA-AtREP2* insertion site. The primers used were MEAP RAD S1: 5′-GATATGTTGG GTCCGTCGG-3′ and MEAP RAD AS1: 5′-CTATGCT CGTCTAGCTAC-3′.

For the PCR analysis spanning the *MEA-AtREP2* helitron region, the PCR conditions consisted of annealing temperature of 55°C (15 sec) and extension time of 30 seconds for 30 cycles.

A series of four different combinations of primer pairs were used for PCR spanning the MEA-ISR region. Primer pair 1 consisted of MEA S40: 5′-GCTATGGACC AGAACATGC-3′ and MEA AS42: 5′-AGGGTTTGCT CTTGAAGTCAG-3′. Primer pair 2 consisted of MEA3′ REP1: 5′-GTGGCTGTAGCTTACGAAAGG-3′ and MEA AS42: 5′-AGGGTTTGCTCTTGAAGTCAG-3′. Primer pair 3 consisted of MEA 3′REP1F: 5′-GTGGCT GTAGCTTACGAAAGG-3′ and MEA 3′REP2R: 5′-GTTTGGATTCGTGATATACACC-3′. Primer pair 4 consisted of MEA S40: 5′-GCTATGGACCAGAA-CATGC-3′ and MEA3′REP2R: 5′-GTTTGGATTCGTG ATATACACC-3′. For the PCR analysis spanning the MEA-ISR, the PCR conditions were annealing temperature of 50°C (15 sec) and extension time of 90 seconds for 30 cycles. *Bam*HI restriction analysis of PCR products was conducted using standard protocols on MEA-ISR PCR products obtained from the following ecotypes: Bla-1, Bla-14, Blh-1, C24, Cit-0, Co-1, Co-2, Col-0, Col-1, Cs22493, Cs22495, Ct-1, Cvi-0, Di-0, Ei-2, Est-0, Est-land, Fr-2, Ga-0, Gr-1, Gu-0, Ha-0, Hodja, Kas-1, Kb-0, Kil-0, Kin-0, KN-0, Le-0, L*er*-0, Lo-1, Me-0, Ms-0, Mt-0, Nd-0, No-0, Nok-0, Np-0, Pa-1, Pog-0, RLD1, Rsch-0, Ru-0, Sah-0, Sf-1, Te-0, Tsu-0, Tu-1, UK-1, Wl-0, Wil-1, Ws, Ws-0, Wt-1, XX-0, and Yo-0. All of these ecotypes produced a PCR product with at least one primer pair combination spanning the MEA-ISR.

Bioinformatics

The *MEA-AtREP2* helitron element was initially identified using the repeat element mapping program Repeat View (http://www.itb.cnr.it/webgene/). The CpG islands were identifed using the CpG islands prediction program available on the Webgene Web site (http://www.itb.cnr.it/webgene/). The *MEA* and *FWA* tandem repeats were identified by the Tandem Repeats Finder program (http://c3.biomath.mssm.edu/trf.basic.submit.html). The large tandem duplication in the *MEA* upstream region was identified from restriction enzyme profiles of the *MEA* locus that exhibited similar restriction patterns indicative of a tandem duplication.

RESULTS AND DISCUSSION

Transposons and Tandem Repeats at the *MEA* and *FWA* Loci

To determine whether the two imprinted loci *MEA* and *FWA* in *Arabidopsis* contained any common structural features that could be associated with epigenetic regulation (i.e., genomic imprinting) we used a range of bioinformatics tools for comparative purposes. The sequences analyzed were generated by the *Arabidopsis* Genome Initiative (2000) and represent the Colombia (Col-0) accession.

In mammals, the regulatory regions of imprinted genes frequently contain a combination of features including tandem repeats associated with differentially methylated CpG islands (Moore 2001). Transposable elements are found in the upstream regions of both *FWA* and *MEA*, although each imprinted gene is proximal to a different type of transposable element in their upstream regions. While *FWA* contains an *AtSINE3* element 980 bp upstream of its start codon, the closest transposable element to the imprinted *MEA* locus is an *AtREP2* helitron found 4363 bp upstream of the *MEA* start codon (Fig. 1A).

The imprinted *MEA* and *FWA* loci also contain tandem repeats (Fig. 1). The *MEA* upstream region contains two tandemly duplicated segments (~1450 bp and ~ 1690 bp) spanning ~3140 bp. However, no analogous large tandem

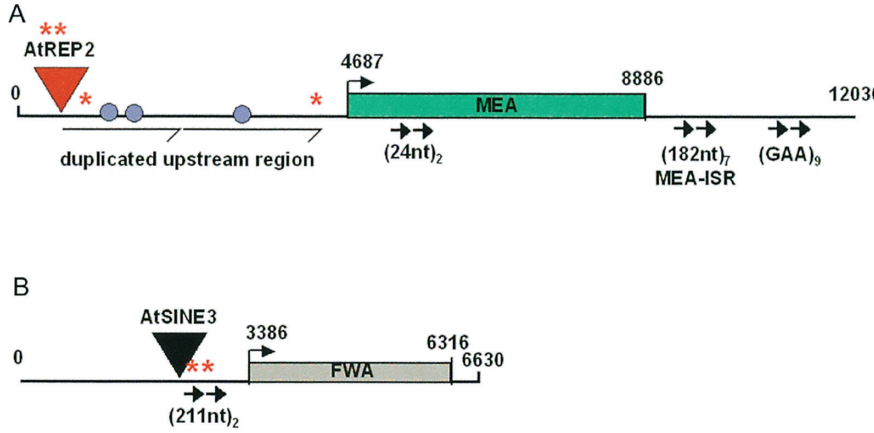

Figure 1. *cis*-elements in known imprinted *Arabidopsis* genes as candidate imprinting control elements (ICEs). (*A*) *MEA* locus (Col-0 accession) with *AtREP2* helitron transposon (*triangle*) and CpG islands (*circles*). Methylated regions upstream of *MEA* as previously reported for stamen and entire seeds (Xiao et al. 2003) are represented as *asterisks*. MEA-ISR refers to MEA intergenic subtelomeric repeat region consisting of eight tandem repeats of 182 nucleotides in Col-0. Other repeats at the MEA locus are a tandem 24-nucleotide repeat and a trinucleotide GAA repeated nine times. (*B*) *FWA* locus (Col-0 accession) with a *AtSINE3* transposon (*triangle*) and tandem 211-nucleotide repeat in the upstream region. Methylated regions as previously reported for embryo, seed coat, and vegetative tissues (Soppe et al. 2000; Kinoshita et al. 2004) are represented by *asterisks*.

duplications are found at the imprinted *FWA* locus. The *MEA* locus contains three different types of tandem repeats: (i) a 24-nucleotide tandem doublet in the third exon of the *MEA* ORF, (ii) a downstream tandem repeat region of seven 182 nucleotide repeats $(182nt)_7$, and (iii) a downstream GAA trinucleotide $(GAA)_9$. The *FWA* locus contains less extensive tandem duplications than the *MEA* locus and contains simply a 211-nucleotide tandem doublet 977 bp upstream of the *FWA* start codon. We also determined whether each of the two imprinted loci contain CpG islands and found that the imprinted *MEA* locus contains 3 CpG islands 3331 bp, 3028 bp, and 1215 bp upstream of its start codon, but the imprinted *FWA* locus contains no CpG islands that we could detect using the GpC island prediction program.

The *AtREP2* Helitron Is Not Required for Imprinting at the *MEA* Locus

The 5′ upstream region of *MEA* contains a nonautonomous *AtREP2*-type *Helitron* transposable element (Kapitonov and Jurka 2001). Helitrons are a novel class of eukaryotic DNA transposons that can transpose by rolling circle replication (Kapitonov and Jurka 2001). Both autonomous and nonautonomous helitrons can be found in eukaryotic genomes. The autonomous rolling-circle (RC) helitrons encode a 5′-to-3′ DNA helicase and nuclease/ligase similar to those encoded by known RC replicons. In addition, numerous nonautonomous RC helitron derivatives can be found throughout some eukaryotic genomes. In *C. elegans*, helitrons (autonomous and nonautonomous) can constitute ~2% of the genome (Kapitonov and Jurka 2001). The *MEA-AtREP2* element is a nonautonomous helitron.

We investigated whether presence of the *AtREP2* transposable element 4363 bp upstream of the *MEA* start codon (Fig. 1A) in the Col-0 and Ler-0 ecotypes is correlated with genomic imprinting at the *MEA* locus taking advantage of the natural variation resources available in *Arabidopsis*. It is known that transposons can accumulate to varying extents between *Arabidopsis* accessions. For instance, a few classes of DNA transposons have been found to be completely absent from some *Arabidopsis* accessions, yet they are prevalent in others. Examples include the low-frequency *Tag1* element absent from Colombia and WS (Frank et al. 1998) and the CACTA family transposons absent from C24 (Kato et al. 2003). However, it is not known whether the RC helitrons display any significant polymorphism between *Arabidopsis* ecotypes.

To identify ecotypes lacking the *MEA-AtREP2* helitron insertion, we used a PCR-based strategy to amplify across the *MEA-AtREP2* insertion site in 33 evolutionarily divergent *Arabidopsis* accessions, four of which are shown in Figure 2. Out of 33 ecotypes screened from an *Arabidopsis* core collection, we identified nine ecotypes where an ~250-bp PCR product was observed (see example in Fig. 2B for Yo-0 and Kb-0). As this size is smaller than the expected ~850 bp, these were candidates where the *MEA-AtREP2* insertion may be absent. To confirm that the *At-REP2* helitron was indeed absent in these accessions, we sequenced of the PCR product spanning the *MEA-AtREP2* insertion site (Fig. 2C). This analysis showed that several accessions, including Yo-0 and Kb-

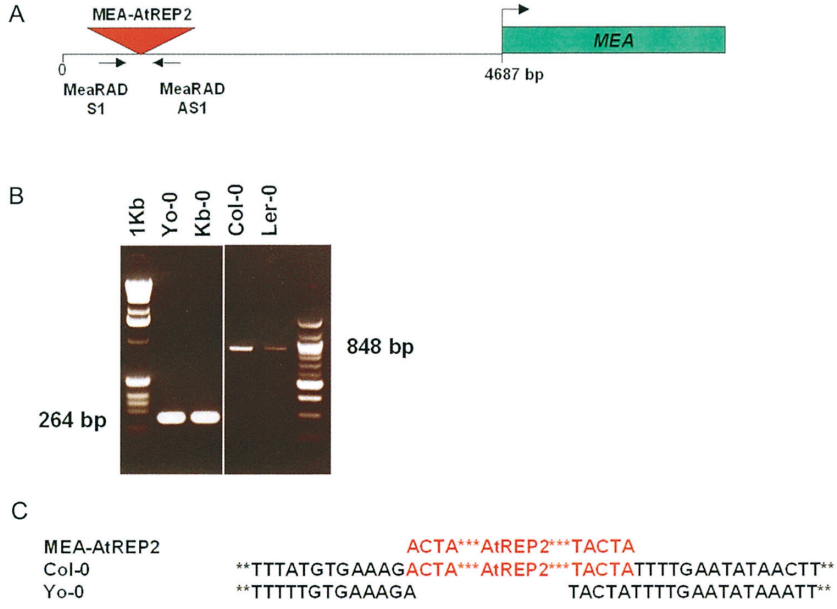

Figure 2. *MEA-AtREP2* helitron is absent in accessions where *MEA* remains imprinted. (*A*) Schematic representation of *MEA* locus indicating *MEA-AtREP2* helitron transposon upstream of *MEA*. Primers (MeaRAD S1 and Mea RAD AS1) spanning the *MEA-AtREP2* insertion are represented by *arrows*. (*B*) Gel electrophoresis of PCR products of four different ecotypes, indicating that this *MEA-AtREP2* is not present in the accessions Yo-0 and Kb-0. (*C*) Sequence analysis across the *MEA-AtREP2* insertion site was used to confirm that the *MEA-AtREP2* helitron was absent in accessions such as Yo-0.

0, completely lack this *AtREP2* helitron.

To link these data with genomic imprinting we investigated whether the accessions lacking the *AtREP2* transposons still contain an imprinted *MEA* locus. A genetic study performed in our laboratory aimed at the identification of modifiers of genomic imprinting at the *MEA* locus within the *Arabidopsis* gene pool (C. Spillane and U. Grossniklaus, unpubl.). To determine whether an accession contained any *cis*- or *trans*-acting modifiers of *mea*-related seed abortion we crossed each accession with a *mea/MEA* pollen donor, and selected F1 hybrids with the genotype *mea/MEA*. Analysis of the F2 seed (aborted: normal) from the selfed *mea*/*MEA* F1 hybrids allowed us to test whether any genetic modifiers of *mea*-related seed abortion were present. For instance, under the assumption that paternally supplied *MEA* activity can rescue *mea* maternal effect seed abortion, the survival of a seed inheriting a mutant *mea* allele from the mother would indicate that the paternally inherited *MEA* allele was active unlike in the L*er* accession. In general, any modifier leading to the survival of seeds that inherited a mutant maternal *mea* allele, or to the abortion of seeds that inherited a wild-type maternal *MEA* allele, distort the 50% seed abortion ratio observed in heterozygous *mea*/*MEA* plants (Grossniklaus et al. 1998). Thus, if we observed a F2 seed abortion ratio of 50% from a selfed *mea*/*MEA* F1 hybrid, the accession contains no modifier, whether paternally, maternally, or zygotically acting. This reasoning applies irrespective of whether the imprint corresponds to the maternal activated state, the paternal silent state, or both. Our analysis showed that the accessions Yo-0 and Kb-0 do not contain any genetic modifiers of genomic imprinting, because all seeds inheriting a mutant maternal *mea* allele abort (Table 1). As both of the ecotypes Yo-0 and Kb-0 lack the *MEA-AtREP2* insertion upstream of the *MEA* locus, this strongly suggests that the *MEA-AtREP2* helitron is not involved in imprinting at the *MEA* locus.

The Repetitive MEA-ISR Tandem Repeats Are Not Required for Imprinting at the *MEA* Locus

Direct tandem repeats have been found proximal to several imprinted genes in mice and humans. The "tandem repeat hypothesis" has been proposed, suggesting that repeats may be important in targeting methylation to differentially methylated regions (DMRs) (Neumann et al. 1995; Lewis et al. 2004). In mammals, evidence for a causal role for tandem repeats in imprinting regulation remains inconclusive (Lewis et al. 2004). In plants, it has been proposed that tandem repeats associated with a SINE transposable element insertion and associated tandem repeats adjacent to the *FWA* gene are the likely cause of imprinting at the *FWA* locus (Lippman et al. 2004).

Downstream from the *MEA* gene we found a conspicuous cluster of short repeats. These 182 nucleotide repeats are also found in 12 other genomic locations in the *Arabidopsis* genome, all of which are also subtelomeric. Hence, Cao and Jacobsen (2002) named the $(182)_7$ repeat region MEA-ISR for intergenic subtelomeric repeat region. They showed that this region attracts high levels of DNA methylation in wild-type strains, namely, 87% at CpG, 47% at CpNpG, and 18% at asymmetric sites. All asymmetric and CpNpG methylation was abolished at the MEA-ISR (and also at the *FWA* 211nt direct repeats) in *drm1, drm2* double-mutant and *drm1, drm2; cmt3-7* triple-mutant backgrounds, while CpG methylation levels remained similar to the wild type (Cao and Jacobsen 2002). Subsequently, Zilberman et al. (2003) demonstrated that the *ARGONAUTE4* (*AGO4*) gene involved in RNA-mediated silencing was also necessary for asymmetric and CpNpG, but not CpG, methylation at the MEA-ISR. In contrast, loss of *AGO4* activity had no effect on asymmetric, CpNpG, or CpG methylation at the *FWA* 211-nucleotide direct repeats.

To determine whether the MEA-ISR region is involved in genomic imprinting at the *MEA* locus, we used a combination of PCR and restriction enzyme-based assays. We screened evolutionarily divergent accessions from an *Arabidopsis* core collection to determine whether any accession lacked the MEA-ISR. For the 56 ecotypes for which we obtained PCR products, our results indicate that the MEA-ISR region has undergone substantial expansions and contractions between accessions, likely because of differences in the number of the 182-nucleotide repeats in each accession (Fig. 3).

In the Col-0 genome, each of the 182-nucleotide repeat regions in the MEA-ISR contains a *Bam*HI (B) restriction site (Fig. 3A). This allowed us to develop a simple assay to test whether each PCR product obtained from primers (e.g., MEA S40 and MEA AS42) spanning the MEA-ISR contained at least one *Bam*HI site. We were interested to identify MEA-ISR-derived PCR products containing no *Bam*HI sites, as these are candidates where the 182-nucleotide repeat region may be absent. *Bam*HI restriction analysis of PCR products from 19 different ecotypes indicated that one or more *Bam*HI sites were present in the MEA-ISR region in all 19 ecotypes tested (results not

Table 1. The Presence of Upstream *MEA-ATREP2* Helitron and Downstream MEA-ISR 182 bp Is Not Necessary for *MEA* Imprinting

Arabidopsis accession	Candidate *cis*-acting element	Aborted F2 seeds	Normal F2 seeds	Total F2 seeds	% aborted F2 seeds	$\chi^{2\,a}$	Best-fit model[b]
L*er*-0	*MEA-AtREP2* and MEA-ISR $(182nt)_7$	215	233	448	47.99	0.362	no modifier
Yo-0	*MEA-AtREP2* absent	1192	1315	2507	47.55	3.019	no modifier
Kb-0	*MEA-AtREP2* absent	277	270	547	50.64	0.045	no modifier
Pa-1	MEA-ISR $(182nt)_1$	1274	1158	2432	52.38	2.768	no modifier

[a]Test of contingency with the expected values for a 1:1 aborted :normal seed ratio corresponding to a "no modifier" model.
[b]For $\alpha < 0.05$ ($\chi^2 = 3.84$, df = 1).

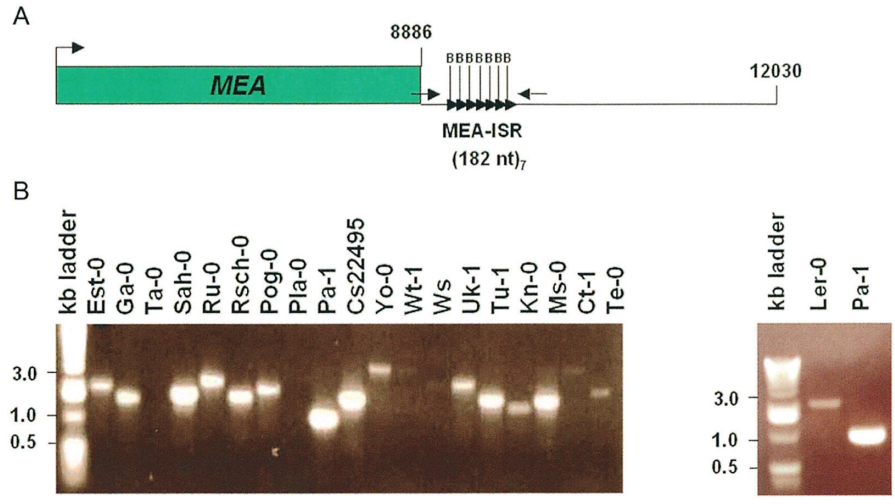

Figure 3. Expansion and contraction of the repetitive MEA-ISR tandem repeats downstream of the imprinted *MEA* locus. (*A*) Schematic representation of MEA-ISR region, where *Bam*HI sites (*B*) are contained in each 182-nucleotide repeat unit. (*B*) Gel electrophoresis of PCR products using primers spanning the MEA-ISR region indicate that the MEA-ISR region undergoes expansions and contractions because of differing numbers of repeat units.

shown). Sequencing of the smallest PCR products allowed the identification of an ecotype (Pa-1) containing only a single MEA-ISR repeat copy (i.e., no repeats of the 182 nucleotides). As the MEA locus remains imprinted in the Pa-1 accession, which contains no modifiers of *MEA* imprinting (Table 1), we conclude that tandem repeats in the MEA-ISR region do not constitute an essential *cis*-acting imprinting control region for the *MEA* locus.

Identification of a Promoter Region Sufficient for Parent-of-Origin-dependent Expression Rules Out a Role for Potential Epigenetic Attractors

To test independently for regions required for imprinted expression of *MEA*, we investigated whether a promoter fragment driving a reporter gene can reproduce the imprinted expression pattern of *MEA*. Transgenic experiments provided evidence that *cis*-acting elements required for imprinting are present in the proximal upstream region of the *MEA* gene. A truncated promoter fragment missing these candidate sequences, but spanning the CpG islands, and comprising the first intron of the *MEA* open reading frame (Fig. 4A), is able to confer imprinted expression on the bacterial *uidA* reporter gene encoding β-glucuronidase (GUS). Reciprocal crosses between transgenic *Arabidopsis* lines carrying the *MEAp:GUS* construct and wild-type plants demonstrated that this promoter fragment is maternally active but paternally inactive in the embryo and endosperm, thereby recapitulating the imprinted expression profile of the endogenous *MEA* gene (Fig. 4B). In agreement with our natural variation studies, these results strongly suggest that the potential epigenetic attractor sites are not required for the parent-of-origin-dependent expression of *MEA*. Neither the *AtREP2* helitron found in the upstream region nor the direct repeats located in 3′ region of the *MEA* gene (both the 187-nucleotide repeats and the GAA trinucleotide repeats) are involved in genomic imprinting at the *MEA* locus.

CONCLUSIONS

In this report we have reviewed the potential role of transposons and repeated sequences in epigenetic gene regulation. There is accumulating evidence that such elements can serve as attractors of epigenetic regulation and that they are involved in gene silencing, paramutation, and genomic imprinting. Recently, a role for the *AtSINE3* transposon in genomic imprinting at the *FWA* locus in *Arabidopsis* was reported (Lippman et al. 2004). In contrast, we could not find evidence for the involvement in genomic imprinting of a transposon upstream, or direct repeat seqences (MEA-ISR and GAA trinucleotide repeats) downstream, of the *MEA* locus. This difference suggests that distinct molecular mechanisms are involved in epigenetic gene regulation by genomic imprinting in plants.

At the *FWA* locus, the *AtSINE3* transposon inserted close to the gene and, in fact, contributes the first two exons to the *FWA* gene (Lippman et al. 2004). Thus, a genetic change led to the formation of a chimeric gene consisting partly of a transposon, which in turn attracts epigenetic modifications. How the transposon is affected differentially in male versus female germ cells, as are several transposons in mammals, is an open question that will attract much attention in the future. At present, very little is known about the function of epigenetic mechanisms during gametogenesis in plants. Of the many *Arabidopsis* mutants affecting epigenetic processes, effects in the gametes have been reported only for mutations in *MET1* (Saze et al. 2003). Our study on the *MEA* locus,

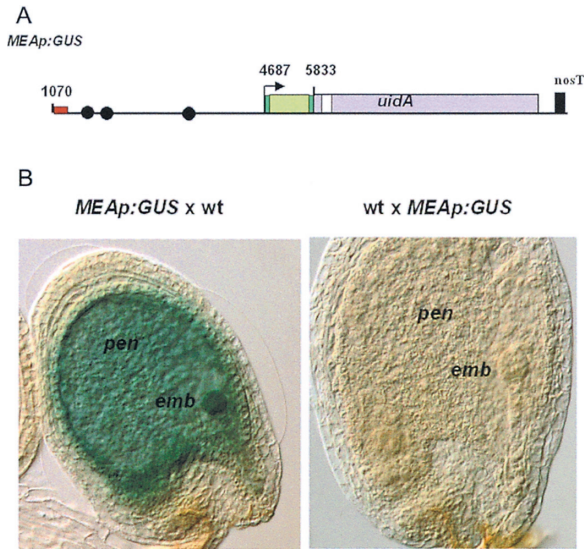

Figure 4. The *AtREP2* helitron and the direct repeats are not required for imprinting. (*A*) The *MEAp:GUS* transgene comprises the *uidA* reporter gene (*purple*) under the transcriptional control of a 4.8-kb *medea* genomic fragment. The fragment spans 3.8-kb promoter sequences lacking the *AtREP2* transposon, except for its distal 70 bp, and incorporates 1 kb upstream open reading frame (*green*; intron in *pale green*), but lacks the direct repeats from the 3′ UTR. (*B*) Imprinted activity of the truncated promoter in developing seeds. *Arabidopsis* lines carrying the *MEAp:GUS* transgene are crossed as female (*left panel*) or male (*right panel*) to wild-type plants (wt). Transgene expression is monitored by the histochemical localization of the GUS enzyme (*blue*) encoded by the *uidA* gene. Abbreviations: pen, peripheral endosperm; emb, embryo at a globular stage.

which ruled out an essential role in imprinting for conspicuous elements such as transposons and repeats, shows that other mechanisms exist as well. The further dissection of *cis*-acting elements required for imprinted expression and of *trans*-acting factors regulating maternal and paternal *MEA* alleles promises to shed light on these alternative mechanisms.

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