# *Imprinting meets genomics: new insights and new challenges*



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**Imprinting meets genomics: new insights and new challenges**

Daniela Pignatta and Mary Gehring*

Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142

*author for correspondence

mgehring@wi.mit.edu

Phone: +1 617-324-0343

Fax: +1 617-258-9872

**Abstract**

Until recently, only a handful of imprinted genes, or genes with parent-of-origin dependent expression patterns, were known in plants. Study of these genes yielded key insights into mechanisms of monoallelic expression and imprinted gene function. The recent application of high throughput sequencing to the study of imprinting has confirmed that many previous findings are relevant on a genome-wide scale. The catalogue of imprinted genes in monocots and dicots now includes a large number of transcription factors, chromatin related genes, and metabolic or hormone biosynthesis enzymes. Interpretation of allele specific expression data remains a challenge, with careful validation of candidate imprinted genes necessary.

## INTRODUCTION

The biology of imprinting is relevant to researchers interested in a wide range of topics including mechanisms of gene regulation, epigenetic reprogramming, and interactions between disparate genomes. Imprinting is an epigenetic phenomenon established during gametogenesis [1]. The expression of an imprinted gene is derived primarily from one parental allele, depending on whether it was inherited from the male or female parent. This phenomenon was initially described in plants [2] but has also been intensively studied in mammals [3].

Endosperm tissue is the primary site of gene imprinting. In flowering plants, the unique process of double fertilization takes place in the female gametophyte, which is surrounded by maternal ovule tissue (Figure 1). Two haploid sperm cells from pollen and two female gametes, the haploid egg cell and the diploid central cell, fuse to simultaneously form the diploid zygote and the triploid endosperm, which nourishes the embryo in a role analogous to the mammalian placenta. Endosperm does not genetically contribute to the next generation but provides nutrients to the growing embryo or germinating seedling.

Many mechanistic and evolutionary aspects of imprinting remain to be fully understood. The recent application of high-throughput sequencing technologies has tremendously increased the number of candidate imprinted genes. Further validation is required to confirm the imprinting status of these genes, their association with differential epigenetic marks, and, ultimately, their role in plant seed development.

## GENOMIC STUDIES OF IMPRINTING

There has been limited understanding of the identity of imprinted genes and their potential role in seed development, with imprinted gene lists in Arabidopsis and maize until recently comprising about 20 genes. Deep sequencing of mRNA libraries derived from reciprocal intraspecific crosses of *Arabidopsis thaliana*, rice, and maize has proven to be an effective strategy to identify genes that are preferentially expressed from one parental allele during seed development [4-9]. These studies have confirmed that imprinting is mostly endosperm-specific and have significantly expanded the number of imprinted genes to between about 50-200 in each species.

Comparisons of candidate imprinted genes identified by independent groups often have limited overlap, as illustrated by two Arabidopsis and two maize studies that utilized the same genetic backgrounds [4,5,7,8]. The Arabidopsis studies identified 208 and 126 candidate imprinted genes, with only 20 genes in common [4]. The seemingly variable

nature of imprinting could in part be due to its transitivity, difference in developmental stage assayed, and bioinformatics and statistical analysis methods employed. Several technical issues can confound the ability to accurately call allelic bias. These include the availability of SNPs, sequencing depth [10], the ability to accurately map reads [11], and contamination from maternal tissues (particularly problematic for small Arabidopsis seeds). Plant studies have used different criteria for calling a gene as imprinted, for example requiring 90% of reads to be from one allele [7], or requiring 5 times greater expression from one allele than the other [8], or simply testing for deviation from the expected 2:1 maternal:paternal ratio in the endosperm [4]. When the two Arabidopsis datasets were analyzed using the same criteria, the overlap increased substantially [4]. Similarly, while only 50 maize genes were in common between the 100 and 179 genes called imprinted by Waters et al and Zhang et al [7,8], 48 of the 129 non-overlapping genes in Zhang had too few reads in the Waters data to asses imprinting, and another 15 genes would have been called imprinted by Waters et al if the filter was relaxed from 90% expression from one parent to 85% (A. Waters, unpublished). Sequencing depth and different statistical tests clearly impact results.

Adding to the complexity, imprinting can be complete (binary), in the case where the expression of a gene is strictly monoallelic, or partial, in the case where both alleles contribute to the expression of an imprinted gene but not according to the expected genome dosage [4]. While the cause is still unknown, partial imprinting might be a reflection of past conflict between alleles (kinship theory) [12] or of a dosage balance, as in the case of transcriptional regulators functioning as part of macromolecular complexes [13]. Imprinting can also be specific to particular strain [6], further highlighting the need to assess imprinting in multiple genetic backgrounds within a species.

Genomics approaches to identify imprinted genes are also being applied in mammalian systems [14-16]. This research faces many of the same challenges as in plants and offers additional useful insight. An exciting paper published in 2010 identified over 1300 candidate imprinted loci in embryonic mouse brain [15], a much greater estimate than previous studies. Recently, Deveale and colleagues repeated the experiment (except E17.5 mice were used instead of E15), performing the same bioinformatic and statistical analyses [17]. Yet only ~13% of novel imprinted genes were in common between the two datasets, about twice as much as expected by chance. Deveale et al found that using data from mock reciprocal crosses to estimate a significance threshold for imprinting effects reduced false positives. Utilizing a similar experimental design in future plant studies may provide additional robustness for identifying imprinted genes.

## THE FUNCTION OF IMPRINTED GENES

Despite tremendous progress in uncovering new plant imprinted genes, only a handful of them have been functionally characterized in the context of seed development. In mammals, the role of imprinted genes in regulating nutrient flow from the mother to the fetus via the placenta is well established [18]. In plants evidence is still lacking, although mutations in several imprinted genes affect the transition from endosperm cell division to cellularization, a developmental step that determines final endosperm and seed size.

A recent study on an imprinted maize gene, *Maternally expressed gene1* (*Meg1*), provides direct evidence that a plant imprinted gene also controls resource allocation [19]. *Meg1* is necessary and sufficient for the establishment and differentiation of the endosperm nutrient transfer cells at the mother-seed interface. Knockdown of *Meg1* leads to small kernels with reduced levels of glucose and fructose. Increasing *Meg1*

dosage produces larger kernels. If one assumes that parental conflict drives imprinting, this finding is partially surprising because as a maternally expressed imprinted gene *Meg1* is expected to restrict rather than promote nutrient allocation to the offspring [12]. Instead, the authors favor a model where the adaptive advantage of *Meg1* imprinting is due to maternal-offspring coadaptation, not conflict [20]. While it is important to note that multiple evolutionary models could explain the evolutionary advantages of imprinting, *Meg1* represents the first evidence of an imprinted gene directly promoting embryo nourishment, consistent with a placenta-like function as in mammals.

Arabidopsis candidate imprinted gene lists are enriched for particular molecular functions. Among paternally expressed imprinted genes (PEGs), transcription factors and chromatin-related proteins are enriched [4]. Examples of imprinted genes include 5-methylcytosine binding proteins, histone lysine methyltransferases, a Class IV homeodomain transcription factor, and several members of the MADS-box transcription factor family [4-6]. Interestingly, different members of the HDG and MADS families are maternally expressed imprinted genes (MEGs). Multiple hormone biosynthesis and response genes, including members of the auxin, ethylene, and jasmonate families, are either MEGs or PEGs. Although many of these genes have well characterized functions at other stages of development (e.g. *EIN2*), the function of most of these genes during seed development has not been described.

While the list of imprinted genes can vary significantly even within a species because of differences in experimental design and analysis, there are a few examples of imprinting conserved between species separated by over a hundred million years of evolution, in addition to the previously identified Polycomb group genes (Table1). Conserved genes are primarily expressed specifically in the endosperm and may represent key regulators of seed development. It is currently unknown whether the molecular mechanism of imprinting at individual loci is the same, although many are associated with differentially methylated regions (Table 1).

## MECHANISMS OF IMPRINTING

Imprinting is under the regulation of at least two non-mutually exclusive mechanisms: DNA methylation and maternal Polycomb repressive complex 2 (PRC2) activity (Figure 2). *MEDEA* and *FWA*, two MEGs, and *PHERES1,* a PEG, are the most extensively studied endosperm imprinted genes in terms of mechanism of monoallelic expression. By performing expression analysis on seeds with mutations in known regulators of imprinting, many more imprinted genes regulated like *MEA*, *FWA*, or *PHE1* were discovered [5,6].

### DNA methylation

Imprinted expression is often associated with differential DNA methylation of the parental alleles. The epigenome is shaped by counteracting DNA methyltransferases and 5-methylcytosine DNA glycosylases, which establish or maintain DNA methylation and remove 5-methylcytosine from DNA by base excision repair, respectively [21]. The 5-methylcytosine DNA glycosylase DEMETER (DME) is expressed in the central cell and is responsible for active demethylation [1]. Consequently, the endosperm is characterized by genome-wide hypomethylation of maternally-inherited DNA, particularly at short transposable elements and repeats, many of which reside near genes [4,22].

About a third of imprinted genes, particularly those that encode regulatory proteins, are associated with differentially methylated regions. These are enriched in Helitron TE sequences [6,22]. For some genes, like *FWA*, methylation likely controls expression by occluding the promoter (Figure 2A). For most loci the functional relationship between methylation dynamics and gene expression remains to be determined. Interestingly, many endosperm imprinted genes are also expressed in pollen [6,22]. This likely reflects expression in the vegetative nucleus, consistent with the loss of methylation in that cell [23].

A screen to identify genes required for expression of an imprinted reporter transgene identified *STRUCTURE SPECIFIC RECOGNITION PROTEIN1* (*SSRP1*), a gene encoding a high mobility group protein that is part of the FACT histone chaperone complex [24]. Maternally inherited mutations of *SSRP1* reduce DNA methylation of SINE-related repetitive elements 5' of *FWA* (Figure 2B). The mutant endosperm has higher methylation levels in all the C contexts compared to wild type, suggesting that SSRP1 is required for DNA demethylation and activation of the *FWA* maternal allele in the central cell, although this may be an indirect effect [24].

**Polycomb group**

Reduced DNA methylation levels in the endosperm might enable targeting of Polycomb group proteins to selected loci, catalyzing trimethylation of lysine 27 on histone H3 (H3K27me3), a hallmark of silent chromatin [25]. In plants, the maternal PRC2 complex comprises the genes *FERILIZATION INDEPENDENT ENDOSPERM* (*FIE*), *FERILIZATION INDEPENDENT SEED 2* (*FIS2*), *MEDEA* (*MEA*) and *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*) (Figure 2C). The FIS PcG complex represses autonomous replication of the central cell, while the sporophytically active PcG proteins suppress seed coat development in the absence of fertilization [26]. Hsieh *et al*. found twenty MEGS, enriched in cellular metabolism and signaling pathways, that exhibited activation of the paternal allele when *fie* was inherited maternally. For some genes expression of the maternal allele was also upregulated. This and another study concluded that PRC2 can regulate maternal and paternal alleles of MEGs and maternal alleles of PEGs [5] [6]. In endosperm, paternal alleles of PEGs might be protected from PRC2 repression because of DNA methylation continuously present at those loci [25].

**Other mechanisms**

Other mechanisms could play a yet uncharacterized role in imprinting. These may be long non-coding RNA (nc-RNA) mediated (Figure 2D), as already established in mammals within imprinted clusters [3]. In *Arabidopsis*, the floral repressor *FLOWERING LOCUS C* (*FLC*), a master regulator of flowering, is stably suppressed by prolonged exposure to cold, which promotes the enrichment of tri-methylated histone H3 Lys 27 at *FLC* locus. This process is mediated by the interaction of a long intronic noncoding RNA (named COLDAIR) to the *FLC* locus via recruitment of PRC2 [27]. Further evidence supporting this idea comes from genome-wide surveys of imprinted genes in rice and maize. While in rice non-genic imprinted transcripts could be partially attributed to misannotated transcripts, a more detailed and experimentally supported characterization of 38 nc-RNAs has been reported in maize [8,9]. This has prompted speculation, as in mammals, that the nc-RNAs could be recruiting a repressive complex (e.g. PRC2) to silence one parental allele and/or allow expression of the other [8].

## IMPRINTING OUTSIDE THE ENDOSPERM?

Several studies that examined allele-specific gene expression in the endosperm also assayed expression in the embryo, where little evidence was found for imprinted expression in Arabidopsis, rice, or maize. This fits with the idea that imprinting is restricted to the endosperm, thus obviating the need for epigenetic resetting between generations [28]. Indeed, assays of allele-specific expression in Arabidopsis, maize, and rice seedlings have found no evidence for parent-of-origin dependent expression [29-31].

In an effort to understand when the zygotic genome becomes transcriptionally active, Nodine and Bartel performed mRNA-seq on very young Arabidopsis reciprocal $F_1$ hybrid embryos (1-cell/2-cell, 8-cell, and ~32-cell stages) [32]. In contrast to animal models and a previous plant investigation, which described the embryo as predominantly controlled by the maternal genome [33], Nodine and Bartel found that maternally and paternally inherited genomes contribute largely equally to the early embryonic transcriptome. Apart from this interesting result, they also found a little over a hundred genes that exhibited parent of origin effects in at least one of the three stages investigated. While some of these could represent inherited transcripts from the egg or sperm [34], this finding suggests a transient form of imprinting might be present in the embryo, previously considered an exception [9,35].

Nodine and Bartel's findings [32] indicated that both genomes switch on soon after fertilization occurs [36]. Yet the plant embryo is not fully autonomous and is subject to parental conflicting control. For example, Bayer et al. [34] hypothesized that while the female parent limits nutrient allocation to the offspring, the male parent could influence growth of the suspensor, which is the organ mediating nutrient transfer to the early embryo before nutrients are derived from the endosperm (Figure 1). Whether or not the suspensor-related genes are subjected to imprinting remains unexplored. Like the endosperm, the suspensor does not contribute to the next generation. Thus the epigenome could also be dynamic there without requiring a need for epigenetic resetting for the next generation. It will be interesting to determine if any of the candidate imprinted embryo genes are, more precisely, suspensor-specific genes.

## CONCLUSIONS

It is an exciting time to be studying imprinting. New technologies mean that imprinting studies no longer need be limited to a few model organisms but can be expanded to species with differing modes of reproduction and seed development. A key challenge for the future is to determine the best methods for collecting and analyzing allele-specific expression data to reduce false positives and false negatives. Increasingly sophisticated methods for tissue isolation should ameliorate technical problems associated with contaminating tissues. Discovery of functions and associated phenotypes for new imprinted genes is another key challenge that offers exciting new avenues into epigenetic mechanisms and seed biology.

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**Table 1**: Conservation of imprinted genes among Arabidopsis, rice and maize.

| Annotation | Arabidopsis Gene ID | Expression | Rice Gene ID | Expression | Maize Gene ID | Expression |
|---|---|---|---|---|---|---|
| YUCCA10, flavin monooxygenase | AT1G48910 | PEG [a,b,c] | Os12g08780.1 | PEG [d] | GRMZM2G091819 | PEG [e] |
| VARIANT IN METHYLATION 5 (VIM5) | AT1G57800 | PEG [a,b,c] | Os04g22240.1* | PEG [d] | AC191534.3 | PEG [e] |
| VARIANT IN METHYLATION 1 (VIM1) | AT1G57820 | PEG [a] MEG [c] | Os04g22240* | PEG [d] | AC191534.3_FG003 | PEG [f] |
| ARID-BRIGHT DNA binding domain | AT4G11400 | PEG [c] | Os10g30944 | PEG [d] | GRMZM2G000404 | PEG [f] |

[a] Gehring et al. (2011), [b] Hsieh et al. (2011), [c] Wolff et al. (2011), [d] Luo et al. (2011), [e] Zhang et al. (2011), [f] Waters and Makarevitch et al. (2012) *Os04g2240.1 has been described as homolog both to VIM5 (Luo et al., 2011; Zhang et al., 2011) and VIM1 (Waters and Makarevitch et al., 2012) PEG (paternally expressed gene), MEG (maternally expressed gene)

## FIGURE LEGENDS

**Figure 1**: *Arabidopsis* female and male reproductive structures with the resulting seed. The diploid central cell (CC) and the haploid egg cell (EC) fuse to the two haploid sperm cells (SC) from pollen. The seed, the result of the double fertilization, contains a diploid embryo, connected to the maternal parent through the embryonic suspensor, a triploid endosperm, and is surrounded by a maternal seed coat.

**Figure 2**: Imprinting mechanisms in *Arabidopsis*. A) DNA methylation: the paternal *FWA* allele is silenced via methylation of the 5' SINE-related repeat elements, while the maternal allele is not methylated and therefore expressed. B) DNA methylation – SSRP1: in *ssrp1* endosperm background, *FWA* maternal allele is partially methylated at the 5' SINE-related repeat elements (represented by boxes) so that its expression is reduced. Methylation of the paternal allele is not significantly affected by the mutation. C) PRC2: Expression of the paternal *PHERES1* (*PHE1*) allele depends on the methylation status of downstream regions (3 open boxes). If the regions are methylated, *PHE1* is expressed (as shown for the paternal allele). The lack of methylation on the corresponding regions of the maternal allele makes *PHE1* a target for PRC2 repressor complex (FIE, MEA, MSI1, FIS2). As a result, the maternal allele is silenced. FIE, FERILIZATION INDEPENDENT ENDOSPERM; FIS2, FERILIZATION INDEPENDENT SEED 2; MEA, MEDEA; MSI1, MULTICOPY SUPPRESSOR OF IRA1. D) Long non-

coding RNA: The expression of a gene (gene x) is subjected to regulation by a long intronic noncoding RNA. In this speculative example, the long nc-RNA recruits PRC2 complex to the maternal allele, which is silenced, in contrast to the paternal allele.

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This study takes advantage of high throughput sequencing of cDNA libraries derived from two *Arabidopsis* ecotypes to discover novel genes with parent of origin-specific expression. It substantially expands the list of known imprinted genes in the endosperm, providing insights into their regulation by multiple mechanisms.

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This study identifies a large number of imprinted genes in *Arabidopsis* by deep sequencing RNA profiles of seeds from intraspecific hybrids. Both DNA methylation and PcG-proteins are described as mechanisms underlying imprinting. The role of transposable elements in shaping the evolution of imprinted genes is also discussed.

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This study identifies hundred imprinted genes in the maize endosperm, of which about a fifth are associated with hypomethylation of either one of the two parental alleles. A few examples of conserved imprinting among maize, rice and Arabidopsis are described.

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This study analyzes global allelic gene expression in reciprocal crosses between two maize inbred lines. Imprinted loci, which include both protein coding genes and long noncoding RNAs, are described as clustered and, in some cases, associated with differentially methylated regions.

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This study identifies over 200 candidate imprinted loci in rice seeds, largely confined to the endosperm. The list comprises both genes and intragenic regions, suggesting that non-coding RNAs could possibly be subjected to imprinting. The comparison between rice and *Arabidopsis* imprinted genes shows little overlap.

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**Figure 1. Female and male reproductive structures with the resulting seed**

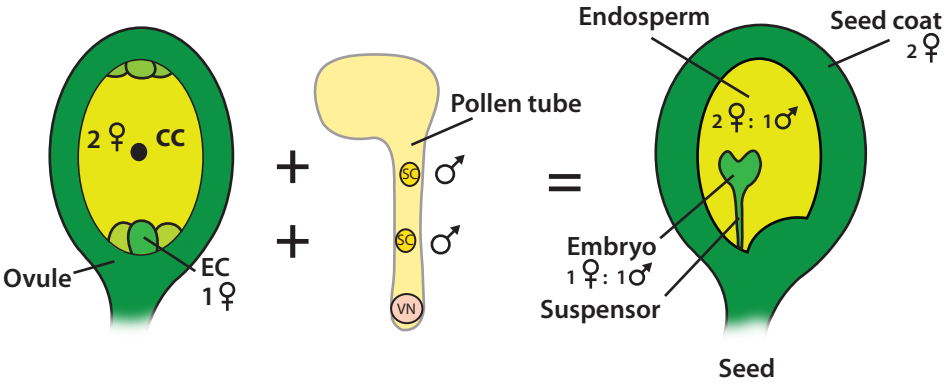
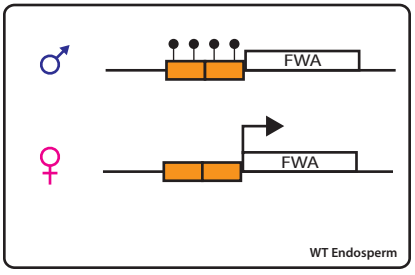
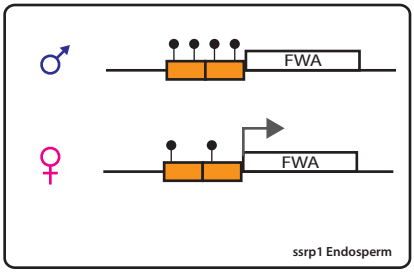



**Figure 2. Examples of imprinting mechanisms in Arabidopsis**

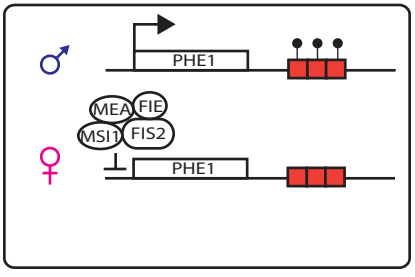
**A. DNA methylation**



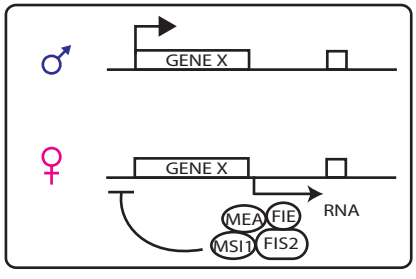

**B. DNA methylation - SSRP1**




**C. PRC2**




**D. long nc- RNA ?**




DNA methylation