REVIEW

Regulation and Flexibility of Genomic Imprinting during Seed Development

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Genomic imprinting results in monoallelic gene expression in a parent-of-origin–dependent manner. It is achieved by the differential epigenetic marking of parental alleles. Over the past decade, studies in the model systems Arabidopsis thaliana and maize (Zea mays) have shown a strong correlation between silent or active states with epigenetic marks, such as DNA methylation and histone modifications, but the nature of the primary imprint has not been clearly established for all imprinted genes. Phenotypes and expression patterns of imprinted genes have fueled the perception that genomic imprinting is specific to the endosperm, a seed tissue that does not contribute to the next generation. However, several lines of evidence suggest a potential role for imprinting in the embryo, raising questions as to how imprints are erased and reset from one generation to the next. Imprinting regulation in flowering plants shows striking similarities, but also some important differences, compared with the mechanisms of imprinting described in mammals. For example, some imprinted genes are involved in seed growth and viability in plants, which is similar in mammals, where imprinted gene regulation is essential for embryonic development. However, it seems to be more flexible in plants, as imprinting requirements can be bypassed to allow the development of clonal offspring in apomicts.

INTRODUCTION

The diploid phase of the life cycle is dominant in the majority of multicellular organisms. As a result, deleterious recessive mutations are masked during the diploid phase (Otto and Goldstein, 1992), implying an equally important role of both parental alleles. While this statement is true for most genes in the genome, imprinted genes represent an exception because only one parental allele is expressed while the other remains silent. Several theories have been proposed to explain the evolution of this epigenetic phenomenon, which is found in organisms as evolutionarily divergent as mammals and flowering plants (reviewed in Haig and Westoby, 1989; Hurst and McVean, 1998; Baroux et al., 2002; Gutierrez-Marcos et al., 2003; Wilkins and Haig, 2003; Feil and Berger, 2007; Moore and Mills, 2008). Because genomic imprinting often results in parent-of-origin–specific effects on the growth of the embryo and extra-embryonic tissues in mammals and plants, the parental conflict theory provides one of the most widely accepted explanations.

Both mammals and flowering plants use a common reproductive strategy (i.e., they share a placental habit). The embryo is embedded and nourished by sexually derived, extra-embryonic tissues: the placenta in mammals and the endosperm in plants. The mammalian placenta and embryo are derived from the same fertilization event and embryonic cells partition early into an inner cell mass, which forms the embryo, and the trophoderm, which will participate in the formation of the placenta. By contrast, the plant embryo and endosperm derive from two distinct fertilization events involving two female gametes, the egg and the central cell, and two sperm cells. The central cell is homodiploid and thus contributes two maternal genomes to the triploid endosperm, whereas only one genome is of paternal origin. This genetic peculiarity is of importance for seed formation and considerably complicates the interpretation of parent-of-origin–specific effects during seed development (reviewed in Birchler, 1993; Spillane et al., 2002; Dilkes and Comai, 2004; von Wangenheim and Peterson, 2004).

Genomic imprinting is conveyed by an epigenetic, parent-of-origin–specific mark (the imprint), which leads to the differential expression of the parental alleles. In mammals, where genomic imprinting is best understood, the imprint is set during gametogenesis, interpreted and maintained during development, and erased and reset in the germ line for the next generation. Genomic imprinting seems to irreversibly set the epigenetic state of certain parental alleles during gametogenesis in animals. As a result, it prevents normal development of gyno- and androgenotes, which carry two maternal or paternal genomes, respectively. Genomic imprinting in plants shares some common principles of regulation with animals and shows some versatility. For instance, it is modulated by parental genomic dosage (Erilova et al., 2009; Tiwari et al., 2010) and might be abrogated in successful hybridization events (Josefsson et al., 2006; Walia et al., 2009), reminiscent of the disruption of genomic imprinting at some loci in interspecific crosses of rodents in the genus Peromyscus (Vrana et al., 1998). Lastly, apomixis, the asexual reproduction through seeds without paternal contribution, likely requires a bypass of genomic imprinting at least in some species (Koltunow and Grossniklaus, 2003; Grossniklaus, 2009).
GENOMIC IMPRINTING AND THE INTRAGENOMIC PARENTAL CONFLICT THEORY

Reciprocal crosses of maize (Zea mays) varieties with differently colored kernels led to the discovery of gene-specific imprinting. In 1970, Kermicle demonstrated that full kernel pigmentation depends on maternal inheritance of the R1 gene, which regulates anthocyanin biosynthesis in the endosperm. By contrast, a mottled pigmentation results when R1 is inherited paternally (Kermicle, 1970). Through a series of elegant genetic experiments, Kermicle could show that this difference in phenotype is due neither to cytoplasmic inheritance nor to a dosage effect in the endosperm but depends solely on the parental origin of R1. The implication of this work was not widely recognized at the time. However, over a decade later, the importance of genomic imprinting was highlighted by nuclear transfer assays in mouse oocytes, showing that both parental genomes are required for normal development of the embryo and, thus, for successful reproduction. Embryonic development cannot be completed if an enucleated egg cell receives only one parental genome (gyngenote, 2m:0p) or two male pronuclei (androgenote, 0m:2p) (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984). Because uniparental disomies of some chromosomes develop normally while some maternal duplications cannot rescue the corresponding paternal deficiencies and vice versa, Cattanach and Kirk (1985) concluded that the entire genome is not subject to parental effects but that imprinted loci reside in specific chromosomal regions. This suggests that the two parental genomes are not equivalent and one genome of each parent is required to complete development, with abnormal expression of imprinted genes underlying the incomplete development of andro- and gynogenotes. Effects of stored components in the cytoplasm of the egg or dosage effects can be excluded, since cytoplasm and parental dosage, except for that of imprinted genes, is the same in both cases.

In placental mammals, mutations in many imprinted genes cause placenta and embryo growth defects in a parent-of-origin-specific manner (DeChiara et al., 1991; Lau et al., 1994; Tycko and Morison, 2002). Similarly in flowering plants, mutations in the methylated promoter regions of Arabidopsis FWA control region seems to control imprinted expression (Kinoshita et al., 2007), the helitron in the FERTILISATION-INDEPENDENT SEED2 (FIS2) induce proliferation defects during seed development (Grossniklaus et al., 1998; Kinoshita et al., 1999; Kiyosue et al., 1999; Vielle-Calzada et al., 1999; Luo et al., 2000; Ingouff et al., 2005b). These defects are consistent with a role of genomic imprinting in a parental conflict over resource allocation from mother to offspring. In mammals and seed plants, postfertilization nutrient provision is at the cost of the mother only, a situation that reduces the resources available for future offspring. Thus, in polygamous organisms with a placental habit, maternally expressed genes are expected to favor parsimonious distribution of nutrients and to antagonize paternally expressed growth factors. In this scenario, growth-restricting genes evolved to be under maternal control, whereas growth-promoting genes are expected to be under paternal control (Haig and Westoby, 1989; Haig and Graham, 1991; Moore and Haig, 1991).

In maize, deletions of specific chromosomal arms reduce kernel size and viability when inherited paternally. These defects cannot be rescued with a higher, maternal dosage of the missing chromosomal arm (reviewed in Kermicle and Alleman, 1990). Thus, genes located on the deleted chromosomal arm may only be expressed from one parent and cannot be rescued by inheriting a compensating dose from the other parent. In addition, increasing the dosage of one parent in Arabidopsis thaliana affects endosperm growth and, consequently, seed size. Normal endosperm development requires a maternal:paternal genome ratio of 2:1 (2m:1p) in many species (Lin, 1984). In some Arabidopsis accessions, however, deviations of this ratio are tolerated, and crosses between different ploidy lines can produce viable seedlings. Consistent with the predictions made from the parental conflict theory, a cross between a tetraploid mother and diploid father (4n × 2n) produces small seeds, whereas the reciprocal cross (2n × 4n) produces larger seeds (Scott et al., 1998). However, these parent-of-origin effects in seeds derived from interploidy crosses are still complex to interpret than the nuclear transfer experiments in mice. Although imprinting of growth regulators likely contribute to the phenotypes, these experiments do not allow precise differentiation from other effects, such as the dosage of cytoplasmically inherited products (Birchler, 1993; Dilkos and Comai, 2004; von Wangenheim and Peterson, 2004) and the role of surrounding maternal tissues (Garcia et al., 2005; Dilkos et al., 2008). The effects on seed size thus likely result from an interplay between cytoplasmic effects, dosage effects, and genomic imprinting.

Alternative theories have been proposed to explain the evolution of genomic imprinting and are reviewed elsewhere (Garner et al., 2008; Moore and Mills, 2008). One of these proposes that genomic imprinting evolved to prevent parthenogenesis, explaining why gynogenotes and androgenotes fail to complete development (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984; Solter, 1988; Varmuza and Mann, 1994). Consistent with this theory, screens for autonomous endosperm development in Arabidopsis revealed, among others, two imprinted genes, namely, MEA and FIS2 (Chaudhury et al., 1997; Kiyosue et al., 1999). However, this phenotype is also observed in mutants affecting nonimprinted Polycomb group genes, and mutation or downregulation of these does not seem to be responsible for parthenogenesis in apomictic Hieracium (Rodrigues et al., 2008).

It was also hypothesized that genomic imprinting evolved as a consequence of repressing foreign DNA, namely, transposable elements (TEs) (Barlow, 1993). In Arabidopsis, methylated pro-moter regions of MEA and FWA coincide with TEs (Lippman et al., 2004; Spillane et al., 2004; Gehring et al., 2006; Kinoshita et al., 2007), but the presence of TEs is not necessarily causally related to imprinted expression. While the SINE-related sequence in the FWA control region seems to control imprinting FWA expression (Kinoshita et al., 2007), the helitron in the MEA promoter is dispensable for imprinting (Spillane et al., 2004), as are the direct repeats downstream of MEA, despite being differentially methylated in the endosperm (Spillane et al., 2004; Gehring et al., 2006). The finding that TEs are hypomethylated in the genome of Arabidopsis endosperm compared with that of the embryo (Gehring et al., 2009; Hsieh et al., 2009) indicates a potential involvement of TE-related sequences in regulating imprinted loci.
DEFINING IMPRINTED LOCI: MEETING THE STANDARDS

Imprinting is defined by the differential expression of parental alleles in the same nucleus. Typically, mammalian imprinted genes have been identified using allele-specific measurements in somatic cells of either transcript levels, DNA methylation, reporter gene activity, or mutant analyses following reciprocal crosses. Imprinted and potentially imprinted genes in flowering plants have been identified using similar approaches (Table 1). Most of the imprinted and potentially imprinted genes have been qualified as imprinted based on differential levels of parental transcripts and/or detection of a reporter gene when inherited through one parent but not the other. Imprinting (de novo monoallelic expression after fertilization) but also cytoplasmic inheritance of maternal or paternal transcripts can explain these observations (Grossniklaus et al., 1998; Vielle-Calzada et al., 2000; Bayer et al., 2009). Thus, an analysis of steady state levels of parental transcripts is only sufficient to demonstrate imprinting if the candidate gene is not expressed prior to fertilization in the gametes, as is the case for the Arabidopsis genes PHEREST1 (PHET1) and FORMIN HOMOLOGS (FHS) and, in the embryo, for the maize gene Maternally expressed in embryo1 (Mee1) (Köhler et al., 2005; Fitz Gerald et al., 2009; Jahnke and Scholten, 2009; Wuest et al., 2010). Alternatively, parent-of-origin-specific expression that increases after fertilization provides evidence for genomic imprinting in addition to parental transcripts potentially inherited from the gametes, whose abundance cannot increase. However, such analyses require quantitative methods and, importantly, proper internal standards that are stably expressed during development in the fertilization products only. This is because carpel cell number is stable during silique development and growth solely depends on cell expansion (Vivian-Smith and Koltunow, 1999). In addition, integument cells proliferate only early in seed development, whereas later on, cell elongation accounts for integument growth (Garcia et al., 2005). Thus, internal standards expressed in all cells of the developing silique do not allow a precise quantitation of mRNA levels. Reliable quantitation requires either standards that are expressed in the fertilization products only or experiments using isolated tissues, the latter being technically demanding at early stages. Thus, monoallelic de novo transcription of candidate imprinted loci is best demonstrated by (1) nascent transcript detection using RNA in situ hybridization, as shown for the imprinted MEA gene in the endosperm (Vieille-Calzada et al., 1999), (2) absence of expression prior to fertilization (Köhler et al., 2005; Fitz Gerald et al., 2009; Jahnke and Scholten, 2009; Wuest et al., 2010), or (3) nuclear run-off transcription assays, which are not possible at early stages due to the limited material available. To remain consistent with previous literature, we discuss both imprinted and potentially imprinted genes (i.e., for which monoallelic expression has not been demonstrated unambiguously) in the following sections.

Furthermore, defining the primary imprint remains a challenging task that requires epigenetic profiling of the candidate loci in isolated male and female gametes, as first performed in maize for Fie1 and Fie2 (Gutiérrez-Marcos et al., 2006). To date, a functional verification showing that the identified mark is indeed necessary for imprinted expression has only been performed for the FWA locus in Arabidopsis (Kinoshita et al., 2007). Importantly, defining the epigenetic profile several days after pollination (DAP) is not sufficient because epigenetic marks can be highly dynamic (Gutiérrez-Marcos et al., 2006; Jahnke and Scholten, 2009) and the marks maintaining imprinting may be different from the primary imprint(s). Although challenging, recent progress in isolating plant gametes and zygotes (Dresselhaus et al., 1999; Engel et al., 2003; Gutiérrez-Marcos et al., 2006; Ning et al., 2006; Hermon et al., 2007; Wuest et al., 2010) should allow a better characterization of the differential epigenetic states of imprinted alleles.

MOLECULAR AND CELLULAR FUNCTIONS OF IMPRINTED GENES DURING SEED DEVELOPMENT

To date, 11 genes in Arabidopsis and 11 genes in maize have been reported as imprinted or potentially imprinted (Table 1). They have been identified on the basis of parent-of-origin-specific effects on seed development (Kermicle, 1970; Grossniklaus et al., 1998; Kinoshita et al., 1999; Vieille-Calzada et al., 1999; Luo et al., 2000), differential transcript levels in interlopolyd or interaccesion crosses (Chaudhury and Messing, 1994; Lund et al., 1995a, 1995b; Gutiérrez-Marcos et al., 2004; Tiwari et al., 2008; Jahnke and Scholten, 2009), or differential DNA methylation levels between embryo and endosperm (Gehring et al., 2009). The latter is not an imprinting criterium per se but may strongly favor the identification of imprinted genes expressed in the endosperm. This study led to the identification of 50 candidate imprinted loci (Gehring et al., 2009), but future investigations are needed to establish whether or not they are indeed regulated by genomic imprinting.

Imprinted genes encode a wide range of molecular functions, ranging from the regulation of pigmentation, protein storage, transcriptional regulation, chromatin modification, and cytoskeletal function to mRNA regulation (Table 1). For instance, five recently described potentially imprinted genes, for which only transcripts from one parental allele were detected in the endosperm (Gehring et al., 2009), encode transcription factors of the homeodomain and MYB class (Table 1). The function of these genes is currently unknown, and future studies will show whether they have parent-of-origin-specific roles during endosperm development. Because most imprinted loci were identified recently, little is known about their role during development, except for four genes in Arabidopsis. Mutations in either of the two Polycomb group genes MEA and FIS2 confer maternal effects on seed development, displaying proliferation defects in the endosperm with and without fertilization (Chaudhury et al., 1997; Grossniklaus and Vieille-Calzada, 1998; Grossniklaus et al., 1998; Kiyosue et al., 1999; Ingouff et al., 2005b). The seeds containing embryo and endosperm derived from mutant gametes eventually abort. Their development is delayed such that embryos derived from mutant eggs only reach the late heart or sometimes torpedo stage, while their wild-type siblings complete embryogenesis (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999; Ingouff et al., 2005b).
Table 1. Imprinted Genes in Arabidopsis and Maize

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Cellular Function</th>
<th>De Novo Transcription</th>
<th>Parent-of-Origin–Specific Effect for</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mRNA Levels</td>
<td>Reporter Activity</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MEA</td>
<td>PcG complex protein</td>
<td>Cell proliferation (embryo, endosperm)</td>
<td>Yes</td>
<td>Yes</td>
<td>H3K27me3, DNA-me</td>
</tr>
<tr>
<td>FWA</td>
<td>HD-ZIP transcription factor</td>
<td>Flowering time regulationa</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>PHE1</td>
<td>MADS box transcription factor</td>
<td>Not known</td>
<td>Yesb</td>
<td>Yes</td>
<td>H3K27me3, DNA-me</td>
</tr>
<tr>
<td>FIS2</td>
<td>PcG complex protein</td>
<td>Cell Proliferation (embryo, endosperm)</td>
<td>n.d.</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MPC</td>
<td>Poly(A) binding C-terminal domain</td>
<td>HD-ZIP transcription factor</td>
<td>Not known</td>
<td>n.d.</td>
<td>Yes</td>
</tr>
<tr>
<td>Hdg8</td>
<td>HD-ZIP transcription factor</td>
<td>Not known</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hdg3</td>
<td>MYB transcription factor</td>
<td>Not known</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Myb3r2</td>
<td>HD-ZIP transcription factor</td>
<td>Not known</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>At5g62110</td>
<td>Homedomain-like protein</td>
<td>Not known</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fh5</td>
<td>Formin homolog</td>
<td>Morphogenesis, cellularization (endosperm)</td>
<td>Yesd</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Maize (locus-specific imprinted genes)</td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Fie1</td>
<td>Homolog of AthFIE</td>
<td>Not known</td>
<td>Yesb</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fie2</td>
<td>Homolog of AthFIE</td>
<td>Not known</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Nrp1</td>
<td>Putative transcription factor</td>
<td>Not known</td>
<td>Yesb</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Meg1</td>
<td>Homolog of MEA</td>
<td>Nutrient transfer?</td>
<td>Yesb</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Mez1</td>
<td>Homolog of MEA</td>
<td>Not known</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mee1</td>
<td>Unknown protein</td>
<td>Not known</td>
<td>Yesb</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>Tubulin homolog</td>
<td>Cytoskeleton</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>Zein</td>
<td>Zein protein</td>
<td>Storage</td>
<td>n.d.</td>
<td>Yes</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

The table displays the imprinted and potentially imprinted genes discovered in plants to date. Molecular and cellular functions and investigated parent-of-origin–specific effects, such as de novo gene expression after fertilization, steady state mRNA levels, and imprinted reporter gene expression are listed if known. In addition, phenotypes of the associated mutants and the associated epigenetic marks are specified. Genes in regular font are paternally expressed, whereas genes in boldface are maternally active. n.d., not determined.

*Overexpression phenotype; function in seed development unknown.

*Expression after fertilization only.

*Expression after fertilization only as no expression was detected in the central cell (Wuest et al., 2010).

*De novo expression in the embryo.
MATERNALLY EXPRESSED PAB C-TERMINAL (MPC) display defects in endosperm development. However, they have distinct molecular functions in cytoskeleton and mRNA biology, respectively (Ingouff et al., 2005a; Tiwari et al., 2008; Fitz Gerald et al., 2009). Furthermore, Maternally expressed gene1 (Meg1), an imprinted gene in maize, is specifically expressed in the transfer cells (Gutiérrez-Marcos et al., 2004; Table 1). These cells are involved in nutrient transfer from the maternal tissues to the seed, suggesting a nutrition-related function of Meg1, a proposal that awaits demonstration. Altogether, the known or predicted functions of imprinted or potentially imprinted genes indicate a role in endosperm growth and nutrient transfer to the seed, consistent with the proposed role of genomic imprinting in mediating parental-origin-specific effects on resource allocation.

In contrast with the endosperm, very little is known about the role of imprinted genes during embryo development, although embryonic phenotypes were originally described for mutants of the fis class and in MPC RNA interference lines (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Tiwari et al., 2008). For instance, embryos lacking maternal MEA function overproliferate (Grossniklaus et al., 1998; Luo et al., 2000), a phenotype similar to the embryo overgrowth resulting from, for example, a mutation in the mouse imprinted gene Insulin-like growth factor type 2 receptor (Barlow et al., 1991; Lau et al., 1994). MEA is expressed in the egg cell and/or early embryo, as shown by RNA in situ hybridization (Ville-Calzada et al., 1999; Spillane et al., 2007), reporter gene analyses (Luo et al., 2000; Spillane et al., 2004, 2007; Figure 1A), and RT-PCR on isolated embryos (Figure 1B). Whether the embryonic expression of MEA is imprinted remains a matter of debate. On the one hand, qualitative RT-PCR using a natural polymorphism between the Landsberg erecta and the RLD accession for allele-specific detection of the parental MEA transcripts argues for biallelic expression at 4 to 8 DAP (Kinoshita et al., 1999; Gehring et al., 2006; Erilova et al., 2009). On the other hand, paternal MEA transcripts were not detected in RT-PCR experiments at 2.3 DAP (Ville-Calzada et al., 1999) nor in a time-course experiment of developing seeds using a quantitative real-time RT-PCR assay based on a polymorphism between the mea-2 and the wild-type allele of the same accession (Baroux et al., 2006; Figure 1C). Possibly, the discrepancies between these studies reflect the different accessions used because it is known that hybridization events can interfere with genomic imprinting (Josefsson et al., 2006; Walia et al., 2009).

A role for genomic imprinting, or at least for imprinted genes, in embryogenesis has often been dismissed (Gehring et al., 2004; Jahnke and Scholten, 2009; Julien and Berger, 2009). The embryonic phenotype and expression pattern of MEA indicates yet unknown roles for MEA, possibly in controlling growth regulators in the embryo. Similarly, functional studies will shed more light on the embryonic role of PHE1, which is expressed and regulated by the Polycomb Repressive Complex 2 containing MEA and other FIS class proteins (FIS-PRC2) in both embryo and endosperm (Köhler et al., 2003, 2005) and the Mee1 gene in maize, which also shows imprinted expression in the embryo (Jahnke and Scholten, 2009).

In conclusion, the best characterized imprinted or potentially imprinted plant genes share a role in endosperm development or are at least preferentially expressed in this tissue. When mutated, some of these genes show endosperm growth abnormalities consistent with a proposed role of genomic imprinting in parental conflicts over resource allocation. Nevertheless, additional studies are required to address the function of imprinted genes in the endosperm and, particularly, in the embryo.

**Figure 1.** Expression of MEA in the Arabidopsis Embryo.

(A) Expression of a MEA:GUS reporter gene expressing β-glucuronidase (GUS) under the control of a 3.8-kb promoter (Spillane et al., 2004) in an isolated 16-cell stage embryo. GUS stainings were performed as described by Baroux et al. (2006), except for using only 0.1 mM potassium hexacyanoferrate (II) and 0.1 mM potassium hexacyanoferrate (III) and incubating for 4 d at 37°C. As the half-life of GUS in early embryos is <10 h (R. Baskar and U. Grossniklaus, unpublished data), GUS expression of this stage indicates de novo expression.

(B) RT-PCR on isolated embryos amplifying MEA (34 cycles), as well as ACTIN1 and ACTIN11 (28 cycles each) as controls. Embryos were isolated in a buffer containing 1.6 units/µL RNase Out (Invitrogen) and 1 mM DTT. An inverted microscope was used to find the right stage and a glass capillary to collect the embryos. Conditions for RT-PCR and the primers detecting MEA, ACTIN1, and ACTIN11 are described by Baroux et al. (2006). Images of isolated embryos and corresponding RNA profiles are shown in Supplemental Figure 1 online.

(C) Allele-specific quantitative PCR for MEA on siliques at different time points: before fertilization (BF) and 1 to 4 DAP. No paternal transcript can be detected, suggesting imprinted expression in both embryo and endosperm (Baroux et al., 2006).

**REGULATION OF GENOMIC IMPRINTING: WHAT DO WE KNOW; WHERE DO WE GO?**

Regulation of genomic imprinting in mammals is complex, and several epigenetic mechanisms involving DNA methylation, histone modifications, and noncoding RNAs are recruited to define
the silent versus active state of parental alleles (reviewed in Ideraabdullah et al., 2008; Koerner and Barlow, 2010). Imprinting control elements (ICEs) are cis-regulatory sequences necessary and sufficient to confer imprinted expression. ICEs can function as promoters, enhancers, locus control regions, or insulator elements that control clusters of imprinted genes in a parent-of-origin-specific manner. ICEs are themselves subjected to epigenetic modifications and are usually differentially methylated (Barlow and Bartolomei, 2007). Our understanding of imprinting regulation in plants is much less profound than in animals. For instance, little is known about potential plant ICEs. Nevertheless, imprinting regulation in plants shows some parallels to the regulation of genomic imprinting in mammals. Over the last few years, variations of a predominant model have been developed to describe imprinting regulation in the Arabidopsis endosperm, which largely relies on the specific demethylation of maternal alleles in the central cell. However, we argue that additional models must be developed to take into account data from maize and to describe imprinting regulation in the embryo.

DNA Methylation: Establishing or Interpreting the Imprint?

Parental alleles of imprinted genes must be marked by a primary imprint inherited from the gametes and interpreted in the fertilization products. In principle, the imprint can be on the active or the silent allele, or both alleles can carry distinct marks. Identifying the imprint remains challenging, and nothing is known about the epigenetic status of imprinted loci in the gametes, except for a few loci in maize (Gutierrez-Marcos et al., 2006; Jahnke and Scholten, 2009). Instead, genetic approaches in Arabidopsis have elucidated the control of imprinted expression, suggesting a fundamental role for DNA demethylation and FIS2 in imprinting regulation.

The maintenance DNA-methyltransferase METHYLTRANSFERASE1 (MET1) and the DNA-glycosylase DEMETER (DME) act antagonistically to achieve monallelic gene expression of MEA, FWA, FIS2, and MPC in Arabidopsis (Choi et al., 2002; Kinoshita et al., 2004; Gehring et al., 2006; Jullien et al., 2006a; Tiwari et al., 2008). The current model proposes that DNA methylation is actively removed by the action of DME (Choi et al., 2002; Gehring et al., 2006). This demethylation might be reinforced by passive loss in the central cell due to MET1 down-regulation by RETINOBLASTOMA RELATED (RBR) and its interactor MULTILOCOPY SUPPRESSOR OF IRA1 (MSI1) (Johnston et al., 2008; Jullien et al., 2008). Consequently, gamete-specific demethylation activates the maternal alleles, leading to monallelic expression of MEA, FWA, FIS2, and MPC after fertilization (Figure 2A). When FWA, FIS2, and MPC are inherited from a hypomethylated met1 mutant father, their paternal alleles are expressed in the endosperm (Luo et al., 2000; Kinoshita et al., 2004; Tiwari et al., 2008), while a paternal MEA allele remains silent (Gehring et al., 2006; Jullien et al., 2006a). Thus, hypomethylation is sufficient to trigger biallelic expression of FWA, FIS2, and MPC but not of MEA. This suggests that default methylation by MET1 and specific demethylation of the maternal alleles in the central cell by DME is required and sufficient for imprinting regulation at these loci except for MEA whose regulation is more complex. In fact, specific MEA reporter genes show imprinted expression independent of DME and MET1 activity (H. Wöhmann and U. Grossniklaus, unpublished data), suggesting that additional, yet unknown, factors may be involved in imprinting regulation (Figure 2B).

Recent findings also implicate DNA methylation in the regulation of PHE1, a MADS box gene that is preferentially expressed from the paternal allele (Köhler et al., 2005) and plays a role in seed development (Köhler et al., 2003; Josefsson et al., 2006). A differentially methylated region has been identified outside the 3′ end of the PHE1 coding region, and PHE1 expression is reduced in met1 mutant plants. Furthermore, a PHE1 reporter gene is not expressed when inherited from a father deficient for DOMAINS REARRANGED METHYLASE1 (DRM1) and DRM2 (Makarevich et al., 2008), which encode de novo DNA methyltransferases (Cao and Jacobsen, 2002). Unlike other imprinted genes in plants, the hypermethylated allele is expressed, and DNA methylation seems to be involved in a mechanism leading to the activation of the paternal PHE1 allele.

In maize, most of the candidate imprinted loci are differentially methylated in the endosperm, suggesting that DNA methylation plays a role in regulating imprinted gene expression in this species as well (Figure 2C, Table 1, and references therein). However, this asymmetry in DNA methylation may not be established prior to fertilization for all alleles. Indeed, Fie2 is hypomethylated in both female gametes and the sperm, yet becomes transiently hypermethylated on the paternal alleles in the endosperm only. By contrast, Fie1 is hypomethylated in the central cell but methylated in the egg and sperm cells. After fertilization, the paternal allele is specifically demethylated in the endosperm not correlating with its silent state (Gutiérrez-Marcos et al., 2006; Hermont et al., 2007). Similarly, DNA methylation at the Mee1 locus is highly dynamic. Mee1 shows imprinted expression in the embryo where the maternal allele is activated at 3 DAP, while it is already expressed prior to fertilization in the central cell and subsequently in the endosperm (Jahnke and Scholten, 2009). Maternal expression correlates with a hypomethylated state of Mee1 in both fertilization products (Figure 2D). However, while the paternal methylation state was inherited from the sperm, the maternal state was not, with both female gametes showing hypermethylation. In the endosperm, the maternal alleles are demethylated after fertilization. In the embryo, they undergo demethylation in the zygote while initially remaining transcriptionally silent and then are remethylated during embryogenesis. These observations suggest that DNA methylation is not the primary imprint in these cases; rather, it may reinforce the transcriptionally active versus silent states. Differential DNA methylation presumably is established downstream of other gender-specific epigenetic marks, which remain to be discovered. Furthermore, these experiments clearly illustrate that DNA methylation is highly dynamic and not always correlated with expression. Due to its dynamics, it is not possible to infer the DNA methylation state in the gametes based on analyses performed at later stages of seed development.

Histone Modifications: Establishing and Maintaining Imprints?

The model that MET1 and DME antagonistically regulate genomic imprinting in plants was first developed for MEA (Choi...
et al., 2002) but turned out to be more complex, since the paternal MEA allele is not derepressed if inherited from a met1 mutant father (Gehring et al., 2006; Jullien et al., 2006a). Thus, a different or additional epigenetic pathway must be involved to silence the paternal allele. FIS-PRC2 itself evidently maintains paternal alleles in a silent state since H3K27 methylation marks, which are dependent on PRC2 function, were found 5' and 3' of the MEA gene in siliques 7 DAP and leafs (Gehring et al., 2006; Jullien et al., 2006a). Importantly, seeds inheriting maternal fie or mea mutations show biparental MEA expression (Baroux et al., 2006; Gehring et al., 2006; Jullien et al., 2006b). Similarly, the FIS-PRC2 complex may be involved in maintaining differential expression at FH5 after fertilization (Fitz Gerald et al., 2009). However, since nothing is known about the dynamics of H3K27 methylation in the gametes nor during early seed development, the exact role of H3K27 methylation in imprinting remains elusive.

The FIS-PRC2 complex clearly plays a role in establishing the imprinted state of PHE1. FIS-PRC2 activity in the female gametes represses the PHE1 maternal allele. The repressed state of the maternal allele is maintained after fertilization, while the paternal allele somehow escapes silencing by FIS-PRC2 in the fertilization products (Köhler et al., 2005). How the FIS-PRC2 distinguishes the two parental alleles after fertilization is not known. Parental alleles might be differentially marked by

(A) Imprinting control at the FWA and FIS2 locus. In the central cell (CC), MET1 is thought to be repressed by RBR and MSI1, which should result in a passive reduction of DNA methylation. DME removes DNA methylation marks in the CC. In the egg cell (EC), DME is not expressed and the locus remains silent. In the sperm cells, MET1 methylates and silences the imprinted gene. After fertilization, the maternal alleles are expressed in the endosperm but not in the embryo.

(B) Imprinting control at the MEA locus. In the CC, DME specifically removes the DNA methylation marks. In the EC and potentially in the CC as well (see text), an unknown imprinting factor renders the maternal MEA allele active. For simplicity, the autorepression of the maternal MEA allele is not shown. In the sperm cells, the paternal MEA locus remains methylated and silent through the action of MET1. After fertilization, the silencing of the paternal MEA allele is reinforced by the action of the FIS-PRC2 complex via H3K27 methylation in the endosperm. It remains unclear how MEA expression is controlled in the embryo. The maternal MEA allele is expressed, while the paternal MEA allele is not detected in some accessions, while it is in others.

(C) Imprinting control of Mez1 and Fie1 in maize. The genetic factors controlling imprinted gene expression in maize are not known, but the associated epigenetic marks for active and silent chromatin are well described. In the CC, the DNA methylation marks are removed, but the maternal alleles remain silent. For Mez1, epigenetic marks and expression patterns are not known in gametes but might follow the same model. In the egg cell and the sperm cells, the loci remain methylated and silent. In the endosperm, the active maternal allele is marked by H3 and H4 acetylation and H3K4 methylation. The silent allele is repressed by DNA methylation and H3K27 methylation. Both, Mez1 and Fie1 are not expressed in the embryo.

(D) Imprinting control at the Mee1 locus. The maternal Mee1 allele is active in the endosperm and the embryo. DNA methylation is probably removed after fertilization only, although Mee1 is weakly expressed in the CC. How exactly the maternal alleles are activated is not known. CC, central cell; EC, egg cell.

Figure 2. Imprinting Regulation of Maternally Expressed Genes in Arabidopsis and Maize.
additional histone modifications, as proposed in maize (see below), or by differential DNA methylation (see above) and thus may be distinguishable for the FIS-PRC2 after fertilization.

Additional histone marks might be involved in distinguishing the parental alleles of imprinted genes in maize in addition to differential methylation (Haun and Springer, 2008). Chromatin immunoprecipitation with antibodies against specific histone modifications followed by allele-specific RT-PCR has been used to test the abundance of specific histone marks at the Mez1, Fie1, and Nrp1 loci in maize. As expected, H3K27 di- and trimethylation, both repressive marks (Peterson and Laniel, 2004), are enriched at all three paternally silent alleles. On the other hand, acetylation of H3 and H4 and dimethylation of H3K4, marks associated with active chromatin (Peterson and Laniel, 2004), are enriched on the active maternal alleles (Figure 2C). The presence of these antagonistic histone marks is specific to the endosperm, since no such enrichment was observed in leaf tissue. Future technical advances might make it possible to investigate the epigenetic profiles of these loci in the gametes to determine whether these marks were established prior to fertilization or correspond to maintenance marks that act downstream of the primary imprint. Whether these chromatin modifications also mark the active and silent alleles at imprinted loci in Arabidopsis is yet to be determined.

VERSATILE PLANT IMPRINTING: HOW FLEXIBLE IS IMPRINTING REGULATION IN PLANTS?

A consequence of genomic imprinting is the strict requirement of both parental alleles for the normal development and physiology in mammals. This is best exemplified by human disorders caused by mutations at imprinted loci (Butler, 2009) but also by developmental abnormalities following cloning by somatic nuclear transfer (Niemann et al., 2008). By contrast, certain species of flowering plants produce maternal embryos in the absence of a paternal contribution (apomixis), suggesting a bypass of genomic imprinting requirements in the embryo and sometimes also in the endosperm (Grossniklaus, 2001; Kolturnow and Grossniklaus, 2003). This may be achieved by the absence of functional components regulating maternal silencing, such as the FIS-PRC2 complex, or silencing in sperm cells. Evidence for both has been suggested in the apomictic genus Hieracium (Tucker et al., 2003; Rodrigues et al., 2010). However, other epigenetic and reversible alterations may operate in nonobligate apomicts able to reproduce both sexually and asexually.

Genomic imprinting may also act as a barrier against interspecific hybridization (Bushell et al., 2003; Gutierrez-Marcos et al., 2003) by creating an imbalance in the relative dosage of maternal and paternal growth regulators (Dilkes and Comai, 2004). Consistent with this hypothesis, maternal PHE1 was derepressed in nonviable hybrid seeds resulting from an interspecific cross of two species in the genus Arabidopsis (Josefsson et al., 2006). Interestingly, increasing the chromosomal dosage of the maternal Arabidopsis parent improves hybrid seed viability, possibly by restoring the appropriate balance of maternal repressors of PHE1 and other FIS-PRC2 targets. Similarly, the level of imprinted MEA expression regulates endosperm responses linked to altered parental dosage by reducing maternal MEA expression in response to increased paternal dosage (Erilova et al., 2009). Additional quantitative measurements of imprinting regulators and targets in seeds with different parental ploidy showed alterations of both the relative levels and imprinting states of some, but not all, imprinted genes. However, no simple model of parental dosage-dependent regulation of imprinted loci could be drawn from these measurements (Jullien and Berger, 2010; Tiwari et al., 2010). This indicates that more complex mechanisms of imprinting regulation are involved, possibly reflected by the dynamic changes in epigenetic marks observed at imprinted loci in maize.

CONCLUSIONS AND PERSPECTIVES

Genomic imprinting has a major impact on seed development, both by influencing seed growth and viability. The developmental phenotypes of mutants affecting certain imprinted genes in plants are consistent with predictions made by the parental conflict theory, but other theories might also explain the evolution of genomic imprinting, which may have arisen due to distinct selective pressures at different loci. Either alone or in combination, both DNA methylation as well as histone modifications confered by the FIS-PRC2 complex are involved in imprinting regulation (Choi et al., 2002; Gehring et al., 2006; Jullien et al., 2006a, 2006b). Importantly, DNA methylation is not sufficient to establish imprinted gene expression at all loci described to date. For instance, it is not clear how paternal expression of HDG3 is achieved, as it is maternally hypomethylated in the endosperm (Gehring et al., 2009). Similarly, DNA methylation does not always correspond to the expression state at imprinted loci in maize, and alleles that show differential methylation in the fertilization products but not in the gametes (Gutiérrez-Marcos et al., 2006; Jahnke and Scholten, 2009) must carry yet unknown primary epigenetic marks. The distinction of primary from secondary marks will be a focus of future research. Furthermore, the complexities of imprinting regulation clearly indicate the existence of additional, yet unknown, factors required for imprinted expression. For instance, the potential involvement of non-codingRNAs, which play an important role in imprinting regulation in mammals (Koerner and Barlow, 2010), has not been rigorously investigated.

Further complexity is added by the fact that Mee1 (Jahnke and Scholten, 2009), PHE1 (köhler et al., 2005), and MEA (vielle-calzada et al., 1999; Baroux et al., 2006; Spillane et al., 2007; Figure 1) show imprinted or potentially imprinted expression in the embryo. It is not clear how differential activity of parental alleles at these loci is established. While DME-mediated DNA methylation in the central cell plays a central role for imprinted expression in the endosperm, DME is not expressed in the egg cell (Choi et al., 2002). Unlike in the endosperm, where erasure and resetting mechanisms for imprints are not required because it does not contribute to the next generation, such mechanisms must exist for genes with imprinted expression in the plant embryo. Resetting mechanisms ensure that the epigenetic state of the parental alleles is not inherited from one generation to the next. The gender-specific resetting of imprints occurs in the germ line during gametogenesis in mammals (Reik, 2007; Lees-Murdock and Walsh, 2008), but nothing is known about this
process in plants. Although maternal Mee1 alleles get remethylated during embryogenesis such that both alleles are equally methylated (Jahnke and Scholten, 2009), this does not constitute such a resetting mechanism because it does not lead to a gender-specific distinction of the alleles. Because plants do not have a segregated germ line, the setting of a gender-specific primary imprint can occur only after the lineages for male and female reproductive organs have been separated.

The fact that genomic imprinting in plants is rather versatile and the requirement for a paternal and maternal genome can be bypassed under certain circumstances is important. One fascinating aspect of plant reproduction is the ability of some species to propagate asexually through seeds. Maybe relaxed imprinting requirements were an essential preadaptation for the evolution of apomixis in these taxa. Therefore, apomixis research might benefit from an improved understanding of imprinting regulation and its function in seed development. New technologies allowing the molecular investigation of gametes and improved genome-wide approaches will uncover more imprinted genes and will certainly produce more detailed genome-wide epigenetic maps that add to our understanding of the role and the regulation of genomic imprinting in flowering plants.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Embryo Isolation and RNA Extraction.

**ACKNOWLEDGMENTS**

We thank Valeria Gagliardi for performing the RT-PCR experiment shown in Figure 1B and our colleagues for stimulating discussions. Our work on imprinting mechanisms is supported by the University of Zürich and grants from the Swiss National Science Foundation (to U.G.) and a fellowship from the Roche Research Foundation (to M.T.R.).

Received November 4, 2010; revised December 29, 2010; accepted January 13, 2011; published January 28, 2011.

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