



## Genomic imprinting in plants: observations and evolutionary implications

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### Abstract

The epigenetic phenomenon of genomic imprinting occurs among both plants and animals. In species where imprinting is observed, there are parent-of-origin effects on the expression of imprinted genes in offspring. This review focuses on imprinting in plants with examples from maize, where gene imprinting was first described, and *Arabidopsis*. Our current understanding of imprinting in plants is presented in the context of cytosine methylation and imprinting in mammals, where developmentally essential genes are imprinted. Important considerations include the structure and organization of imprinted genes and the role of regional, differential methylation. Imprinting in plants may be related to other epigenetic phenomena including paramutation and transgene silencing. Finally, we discuss the role of gene structure and evolutionary implications of imprinting in plants.

### Introduction

Genomic imprinting is an epigenetic phenomenon in which the activity of a gene is reversibly modified depending on the sex of the parent that transmits it. Thus, contrary to the expectations of simple Mendelian inheritance, imprinting results in non-equivalent expression of maternally and paternally derived alleles in an individual; usually one of the alleles is suppressed during development. This suppression is correlated with increased cytosine methylation or chromatin-mediated epigenetic silencing (Kooter *et al.*, 1999). In the case of two alleles that have a different inherent phenotype (big 'A' versus little 'a'), the recessive *a* allele may appear to be dominant in an *A/a* heterozygote where the dominant allele *A* is silenced by imprinting. Consequently, the terms dominant and recessive in the usual Mendelian genetic sense are problematic at imprinted loci.

Using the barometer of many college freshman biology textbooks, imprinting is no longer either ignored or viewed as a genetic curiosity, but is now described in the context of its importance during development. The increased attention to imprinting among biologists is in large part due to the recognition, in the 1980s, of its importance during mammalian devel-

opment (Monk, 1988). Also increasing the level of interest, imprinting appears to underlie several human genetic diseases (Clarke, 1990; Hall, 1990; Lalande, 1996; Nicholls, 1998; Tilghman, 1999) and is involved in the proper development of the seed in cereal crops (Kermicle and Alleman, 1990).

Though this review focuses on genomic imprinting in plants, it is instructive first to consider imprinting in other organisms, particularly the mouse, *Mus musculus*. Regardless of whether the discussion of imprinting focuses on mouse, maize, or other species, a number of intriguing questions remain:

- (1) When and how are imprinted alleles modified in the parental germ line?
- (2) Are imprinting 'marks' necessary for genomic imprinting in both animals and plants?
- (3) How do imprints result in parent-specific gene expression during development?
- (4) Is there a set of organism-independent requirements for a gene to show imprinting?
- (5) What is the evolutionary significance of imprinting?

## Historical perspectives

The term 'imprinting' is borrowed from behavioral studies and, in the genetic sense, derives from the studies of Crouse (1960) on parent-of-origin-specific chromosome elimination in the dipteran insect *Sciara*. Other early observations of imprinting in insects include the heterochromatization of the whole paternal set of chromosomes in mealy bugs (Brown and Nur, 1964; Nur, 1990). Another example of whole-chromosome imprinting is the parent-specific X chromosome inactivation in the extraembryonic membranes of rodents (Takagi and Sasaki, 1975; West *et al.*, 1977) and the inactivation of the paternal X chromosome in marsupials (Sharman, 1971). Kermicle (1970, 1978) first described single-gene imprinting, in which silencing affects individual genes, for alleles of the maize *r1* locus that show parent-specific differences in expression during the development of the endosperm of the kernel. One of these imprinted *r1* alleles, *R-r:standard (std)*, specifies a fully pigmented aleurone when inherited from the female parent, but a mottled or nearly colorless phenotype when inherited from the male parent (Kermicle and Alleman, 1990; Figure 1A).

In vertebrates, imprinting of individual mouse and human genes is well documented (Bartolomei and Tilghman, 1997; Reik and Walter, 1998; Brannon and Bartolomei, 1999), as is the imprinting of transgenes in mice (Swain *et al.*, 1987; Chaillet *et al.*, 1991) and zebrafish (Martin and McGowan, 1995a, b). Although androgenetic and gynogenetic haploid fish are abnormal and eventually die, the development of fertile androgenetic diploid zebrafish indicates the absence of essential, paternally imprinted genes, perhaps relating to the absence of large-scale changes in DNA methylation during early development (Corley-Smith *et al.*, 1996; Macleod *et al.* 1999). Nonetheless, parent-of-origin effects on transgenes do occur in zebrafish. In one example, paternally inherited transgenes are more methylated relative to maternally inherited transgenes (Martin and McGowan, 1995a, b).

Imprinting and parental effects are also known in *Drosophila*. Many of these are associated with chromosome rearrangements that show parent-specific position effect variegation of genes in the rearrangements (i.e. Spofford, 1959; 1961; Karpen and Spradling, 1990), presumably due to effects on chromatin structure. A recent paper by Lloyd *et al.* (1999) provides a rigorous demonstration of gene imprinting in *Drosophila*. These authors describe the imprinting of

three closely linked genes on a mini-X chromosome as assessed by silencing when inherited from the male parent, in contrast with wild-type expression when inherited from the mother. They demonstrate that factors including chemicals, the environment, and genetic modifier loci that affect position effect variegation, also influence the somatic maintenance of the imprint, but do not affect the establishment of the imprint. They infer that altered chromatin structure is important in the maintenance of the imprint, but that independent genetic control may be necessary to establish the imprint. As is the case for zebrafish, androgenic and gynogenic *Drosophila* develop normally (Muller, 1958; Fuyama, 1984; Komma and Endow, 1995). Moreover, although imprinting occurs in *Drosophila* as identified in analyses of position effects on genes in chromosomal translocations, it does not appear to affect developmentally essential genes.

## Genomic imprinting in mice and man

### *Non-equivalence of mouse and human maternal and paternal genomes*

The definition of genomic imprinting hinges on the differential expression of maternally and paternally inherited alleles, regardless of organism. Much of the dogma surrounding imprinting in mammals derives from one significant observation: the requirement for the presence of both maternal and paternal genomes to complete embryogenesis. Other organisms, including birds, fish, insects, and amphibians, can be produced from haploid or uniparental diploid zygotes by parthenogenesis (Muller, 1958; Harada and Buss, 1981; Streisinger *et al.*, 1981; Gillespie and Armstrong, 1981; Fuyama, 1984; Martin and McGowan, 1995a, b; Komma and Endow, 1995).

A number of observations indicate the non-equivalence of the maternal and paternal genomes in mice. Nuclear transplantation experiments demonstrate that diploid mouse embryos derived from two maternal haploid genomes fail to develop (Surani and Barton, 1983; McGrath and Solter, 1984). These gynogenotes are characterized by poorly developed extra-embryonic membranes. By comparison, androgenotes (diploid embryos derived from two paternal haploid genomes) also fail to develop, but have more fully developed extra-embryonic structures. Similarly, in man, hydatiform moles typically arise from an egg containing paternally derived nuclei and

no maternal genome contribution; these tumors are characterized by placental cell types (Jacobs *et al.*, 1980).

Using mouse chromosome mechanics, it is possible to construct uniparental segmental disomics to assess parent-of-origin effects for specific chromosomal regions. Through this analysis, which uses translocations to generate mice that inherit one chromosome pair from the same parent, at least seven chromosomal regions in the mouse show non-equivalence of the paternal and maternal genomes (Cattenach and Kirk, 1984). This effectively eliminates the possibility that lack of an extra-genetic contribution of required information by the sperm (cytoplasmic) or homozygous lethal loci (nuclear) caused the developmental failure (Solter, 1988). Thus, using translocations to produce uniparental disomics and nullisomics, and correlating these data with the knowledge of specific, single genes that undergo genomic imprinting, it is important to note that genes, not genomes, are imprinted.

A number of regions of the human genome contain imprinted genes as determined through the analysis of chromosomal deletions. For example, Prader-Willi syndrome results from the deletion of region q11-q13 of chromosome 15 when inherited through the father, whereas Angelman syndrome is associated with a maternally inherited deletion of the same region of chromosome 15 (Jiang *et al.*, 1998). Both syndromes show a range of abnormal phenotypes including mental retardation, as well as obesity in Prader-Willi syndrome, and hyperactivity in Angelman syndrome. Another example that points to the importance of imprinted regions in man is Beckwith-Wiedemann syndrome. This disease, characterized by generalized overgrowth in neonates and increased susceptibility to Wilms' tumor and several other types of cancer, shows sporadic occurrence in uniparental, paternal disomics of a region of chromosome 11 (Junien, 1992).

#### *The underlying basis for mammalian imprinting is differential DNA methylation*

Analyses of imprinted regions of mouse and human chromosomes reveal a large, and growing number of imprinted genes. From the initial report of imprinting of the mouse *insulin-like growth factor-2 (igf-2)* gene in 1991 by DeChiara *et al.*, to a tally of a dozen genes in 1996 (Leighton *et al.*, 1996), there are now over 30 genes known to be imprinted in the mouse genome (Kelsey, 1999).

With a single exception (Casparly *et al.*, 1998), differential DNA methylation of maternal versus paternal alleles occurs in all cases of gene imprinting in mice (Razin and Cedar, 1994; Reik and Walter, 1998; Brannon and Bartolomei, 1999; Feil and Khosla, 1999). Supporting the importance of methylation, imprinting of specific genes is disrupted in methyltransferase-deficient mice (Li *et al.*, 1993). One of the best characterized imprinted regions of the mouse genome contains a cluster of imprinted genes including *Igf-2*, *H19* (producing an untranslated RNA), and *Snrpn* (encoding a *trans*-splicing factor). Molecular analysis of this region revealed the presence of a number of differentially methylated regions (DMR) in, or near, the imprinted genes.

Typically, DMRs occur in or near CpG-rich regions, or islands. These CpG regions are often near short (up to 2 kb) blocks of different types of short directly repeated sequences (Neumann *et al.*, 1995). Though imprinted genes contain DMRs, the actual function of these regions in imprinting remains unresolved. Two DMRs from the mouse *H19* gene act as gene silencers when introduced as transgenes into *Drosophila* (Lyko *et al.*, 1997), an organism that exhibits imprinting (Lloyd *et al.*, 1999), but does not use DNA methylation to control gene expression (Bird and Taggart, 1980; Urieli-Shoval *et al.*, 1982). DMRs clearly are important in the expression of imprinted genes in mouse and man. Interestingly, the *Rasgrfl* gene is imprinted in mouse and rat, and contains a repeat region adjacent to a DMR. The same gene in the closely related rodent, *Peromyscus*, is not imprinted and does not contain the repeat region (Pearsall *et al.*, 1999). These experiments emphasize the involvement of methylation and gene structure in gene imprinting in mammals.

#### *Establishing the imprint*

If differential methylation is the hallmark of virtually all imprinted genes in the mouse, how is the sex-specific mark established during germline development? For a new imprint to be established, the previous genomic imprint first must be erased (Surani, 1998). In the mouse, this occurs in the primordial germ cells during embryonic development (Brandeis *et al.*, 1993; Szabo and Mann, 1995; Shemer *et al.*, 1997; Tada *et al.*, 1997). In the case of maternal-specific imprinting, the imprint is established during oocyte maturation (Kono *et al.*, 1996; Obata *et al.*, 1998; Martineit *et al.*, 1998), whereas the paternal imprint



**Figure 1.** Imprinting of the maize *r1* gene in the endosperm. Imprinting of the *r1* gene in the maize endosperm is demonstrated visually with reciprocal crosses between a colorless kernel *r1* allele (*r-g*) and various colored kernel alleles or epialleles. According to convention, maternal parents are shown on the left side in a cross. A. (upper left) Shown are ears from the crosses involving the imprinting allele, *R-r:std*. Left ear: *R-r:std/R-r:std* × *r-g/r-g*; kernel genotype: *R-r:std*, *R-r:std/r-g*. Right ear: *r-g/r-g* × *R-r:std/R-r:std*; kernel genotype: *r-g*, *r-g/R-r:std*. The mottled phenotype shown by paternally transmitted *R-r:std* is due to imprinting of this *r1* allele. B. (upper right) Shown are ears from the crosses involving the paramutant, imprinting allele, *R-r:std'*. Left ear: *R-r:std'/R-r:std'* × *r-g/r-g*; kernel genotype: *R-r:std'*, *R-r:std'/r-g*. Right ear: *r-g/r-g* × *R-r:std'/R-r:std'*; kernel genotype: *r-g*, *r-g/R-r:std'*. The ' designates a strongly paramutant form of *R-r:std*. This paramutant allele was produced by crossing *R-r:std* twice (two successive generations) with *R-st* and recovering by self pollinating. Both maternally and paternally inherited *R-r:std'* produce less pigmentation than does *R-r:std* as a result of paramutation. The extremely lightly mottled phenotype (nearly invisible) shown by paternally-transmitted *R-r:std'* is the typical expression of the paramutant form of this allele. C. (lower left) Shown are ears from crosses involving the non-imprinting allele, *R-sc:124*. Left ear: *R-sc:124/R-sc:124* × *r-g/r-g*; kernel genotype: *R-sc:124*, *R-sc:124/r-g*. Right ear: *r-g/r-g* × *R-sc:124/R-sc:124*; kernel genotype: *r-g/r-g/R-sc:124*. The mottled phenotype associated with imprinting is absent in the non-imprinting *R-sc:124* allele, even after crossing with *R-st*. Any slight difference in the intensity of the pigmentation in the two crosses is due to a dosage effect in the triploid endosperm. D. (lower right) Shown are ears from crosses in which a weakly paramutant *r1* allele is transmitted maternally with either *Mdr1-w22* or *mdr1-r*, a mutant of the imprinting control gene. Left ear: *R-r:std'/r-g* × *r-g/r-g*. Right ear: *R-r:std'/r-g*, *Mdr1-w22/mdr1-r* × *r-g/r-g*. Kernel genotypes for the right ear: (colorless kernels) *r-g*, *r-g/r-g*, (solid kernels) *R-r:std'*, *R-r:std'/r-g*; *Mdr1-w22*, *Mdr1-w22/Mdr1-w22*, (mottled kernels) *R-r:std'*, *R-r:std'/r-g*; *mdr1-r*, *mdr1-r/Mdr1-w22*.

is established in the male germline prior to meiosis (Kimura *et al.*, 1998; Ogura *et al.*, 1998).

## Imprinting in plants

### *Requirement for maternal and paternal genomes in plants*

Just as the requirement for both maternal and paternal genomes for successful development can be cited as evidence for imprinting in mammals, the existence of viable parthenogenotes in other organisms may be viewed as evidence to the contrary, at least for developmentally essential genes. In plants, maternally and paternally derived haploids develop with a relatively normal body plan, providing clear evidence for the lack of requirement for both parental genomes in the sporophyte. Haploid plants can be produced via anther culture (Chen, 1977; Nitch, 1969), spontaneously (Kimber and Riley, 1963), or in certain genetic backgrounds (Kermicle, 1969; Sarkar and Coe, 1969). Although the sporophyte of both monocots and dicots can develop without the contribution of both a maternal and paternal genome, this is not true for the endosperm where both maternal and paternal genomes are required for successful development. This suggests that genomic imprinting of developmentally essential genes occurs during endosperm development in angiosperms.

### *Double fertilization and endosperm development in maize and Arabidopsis*

Flowering plants undergo double fertilization. Two sperm are produced from a single product of meiosis during microsporogenesis; one fertilizes the egg

mother cell to produce the diploid, embryonic sporophyte; the other fertilizes the diploid central cell to produce a triploid endosperm. It is assumed that the two sperm derived from one meiotic product are genetically identical. Similarly, the egg and the central cell nuclei are assumed to contain identical genomes that differ in ploidy. Thus, although formed by two separate fertilizations, the embryo and the endosperm are genetic twins that differ only by the balance of maternal and paternal genomes.

The endosperm of angiosperms is a specialized tissue that contains polyploid nuclei. In addition, the endosperm is terminally differentiated tissue that does not contribute genetically to the next generation. Development proceeds through a syncytial stage, cellularization, differentiation, and cell death. Only the outermost cell layer contains living cells in mature seeds. In cereal monocots such as maize, the endosperm is a conspicuous tissue composed of proteins and starch for energy. In *Arabidopsis* and most dicots, the endosperm is small, and is absorbed by the embryo during development. Its function is debatable, but it likely is important in directing nutrients to the embryo, with the major energy-storage function assumed by the cotyledons (Hirner *et al.*, 1998; Berger, 1999). In both maize and *Arabidopsis*, nonetheless, endosperm development is crucial for the success of the embryo.

### *Chromosomal imbalance in Arabidopsis*

Interploidy crosses are used to perturb the balance of chromosome sets in *Arabidopsis*. Like other plants, differences in ploidy affect the sporophyte minimally, and, similarly, disrupting the balance between maternal and paternal sets in the endosperm results in abnormal seed. Scott *et al.* (1998) examined the

fertility, seed weight, chromosome numbers, and developmental events of interploidy crosses involving diploid, tetraploid, and hexaploid plants. All interploidy crosses involving hexaploid parents produce inviable seed which were disrupted in number of nuclei formed, timing of cellularization, and development of specific sections of the endosperm. Unlike maize, diploid *Arabidopsis* plants crossed with tetraploid plants produced viable, although abnormal seed with complementary results, as follows. With maternal excess (4:1 maternal/paternal ratio in the endosperm), seed were dramatically reduced in size with the effect on either differentiation or altered rates of mitosis. With paternal excess (2:2 maternal/paternal ratio), larger seed is produced possibly because delayed cellularization may allow additional mitoses. Endosperm failure was viewed as the cause of embryo abortion, with the suggestion that imprinted loci affect seed growth (Scott *et al.*, 1998).

#### *Chromosome imbalance in the maize endosperm*

The best evidence for a requirement of both parental genomes for proper development comes from maize where methods exist for varying the endosperm and embryo karyotypes independently. The *ig1* gene (*indeterminate gametophyte-1*) provides a tool with which to study chromosome set imbalance in the maize endosperm. The predominant phenotype of *ig1*, a high fraction of aborted kernels on *ig1* mutant ears, reflects abnormalities in maternal gametophyte development, resulting in polyembryony, defective microtubule organization, and the production of an indeterminate number of micropylar cells in the embryo sac (Kermicle, 1969; Lin, 1978; Huang and Sheridan, 1996). The *ig1* mutation, then, is a means to produce seeds with aberrant ploidy levels in both the embryo and the endosperm.

In homozygous *ig1* plants, the frequency of haploids among the progeny is increased from  $10^{-3}$  (spontaneous maternal origin) to  $10^{-5}$  (spontaneous paternal origin) to over 3% (Kermicle, 1969). Haploid maize plants of spontaneous origin or from *ig1* lines are associated exclusively with kernels with normal endosperm ploidy (3N) (Kermicle, 1969; Sarkar and Coe, 1969). In addition, Lin (1984) showed that the endosperm of progeny kernels from *ig1* plants always contained both maternal and paternal chromosome sets in spite of a range of ploidy levels in the sporophyte. His cytogenetic survey of chromosome number in defective and normal endosperm

indicates that the ratio of maternal to paternal genomes is crucial (Lin, 1984). Changes in the ratio of 2:1 (maternal/paternal) cause endosperm failure and embryo abortion. Normal endosperms were 2:1 or 4:2. Defective or abnormal endosperm included all other classes from diploid through octaploid. Thus, not only are both maternal and paternal genomes necessary for normal endosperm development, but the ratio of these genomes must retain the normal 2:1 (maternal/paternal) balance.

#### *The small-kernel effect in maize*

Whereas the use of the *ig1* mutation in maize allows varying whole sets of chromosomes with differing parental origin, translocations involving normal (A) chromosomes and supernumerary or B chromosomes can be used to identify genomic regions with parent-of-origin effects. B chromosomes undergo non-disjunction in the second microspore mitosis in pollen at a high frequency (Beckett, 1991). Thus, B<sup>A</sup> translocations, which include a B chromosome centromere with a translocated A chromosome arm, are used to produce paternally duplicate or deficient chromosomal regions for any chromosome arm. For example, the cross 1/1 × B<sup>1</sup>/1<sup>B</sup> will produce a paternal contribution to the endosperm of (1, B<sup>1</sup>), (1, B<sup>1</sup>, B<sup>1</sup>) or (1<sup>B</sup>) with a range of 0–3 paternal chromosome 1 arms. Using genetically marked stocks, eight chromosome arms were identified as having strong, cell non-autonomous effects of parentage, with the deficient paternal class producing a much smaller than normal kernel, down to 50% of the normal weight (Birchler, 1980; Lin, 1984; Birchler, 1993). This phenomenon is termed the small-kernel effect.

It is easy to extrapolate from the small-kernel effect or genome dosage imbalance in the endosperm to genomic imprinting using a model that is analogous to the mouse gynogenote, androgenote, and uniparental disomics. Imprinted genes that are only expressed paternally may cause these parent-of-origin-specific defects in endosperm development (Lin, 1982, 1984). Until affected chromosomal regions are correlated with specific cases of genomic imprinting for single or groups of loci, the small-kernel phenomenon remains a parent-of-origin 'effect'. Other models can also explain these data including Birchler's alternative by which an imbalance of dosage-sensitive genes or regions causes the small-kernel phenotypes (Birchler and Hart, 1987; Birchler, 1993). Nonetheless, because paternally deficient regions cause a reduction in kernel

size, these results are cited as possible evidence for an interesting parallel between plants and mammals in support of the conflict theory for the evolution of imprinting (Haig and Westoby, 1989; Moore and Haig, 1991).

### Single-gene imprinting systems in plants

#### *The maize r1 locus*

The first example of single-gene imprinting involved the *r1* allele, *R-r:std*, which specifies a fully pigmented aleurone when inherited from the female parent but a mottled or nearly colorless phenotype when inherited from the male parent. Imprinting at *r1* is demonstrated visually in reciprocal crosses between colored aleurone and colorless aleurone alleles (see Figure 1A and 1B).

*r1* is a complex locus located on chromosome 10 of maize, controlled by 5' regulatory information as well as the epigenetic phenomena of imprinting and paramutation. *r1* alleles or haplotypes can be composed of one to five tightly linked homologous genes in either direct or indirect orientation (Robbins *et al.*, 1991; Eggleston *et al.*, 1995). Among a large collection of naturally occurring variant alleles, there are several unique structures among *r1* haplotypes spanning up to 200 kb (Walker *et al.*, 1995). Most of these *r1* alleles are composed of multiple genes (M. Alleman, unpublished information). Individual *r1* genes encode a basic helix-loop-helix (b-HLH) transcription factor that determines the developmental timing and tissue-specificity of structural genes in the anthocyanin biosynthetic pathway (Dellaporta *et al.*, 1988; Ludwig *et al.*, 1989; Dooner *et al.*, 1991). The *r1* genes within a given haplotype are sometimes associated with different tissue-specific patterns of expression. For example, the *R-r:std* complex is composed of a *P* gene (*plant color*), a *q* (*quiescent*) promoter pseudo-gene, and an *S* (*seed color*) sub-complex composed of two genes (*S1* and *S2*) in inverted arrangement around a central promoter (Robbins *et al.*, 1991; Walker *et al.*, 1995).

Direct evidence that the mottled phenotype of paternally transmitted *R-r:std* is not simply a dosage effect was obtained by Kermicle (1978). He used *B-A* translocations involving chromosome 10 in which the *R-r:std* or *r-g* (colorless) allele was linked to the *B* chromosome centromere [*B•10 (R-r:std)*] or [*B•10 (r-g)*] to produce segmental duplications or deletions

of the chromosome 10L arm. The *R-r:std* allele is denoted as 'R' and the *r-g* allele as 'r' in the following discussion. The maternal alleles are listed first, by convention, as (m, m/p). Crosses between plants carrying translocated and normal chromosomes produced the endosperm genotypes: (*r, r/R, R*) and (*R, R/r, r*). The genotype (*r, r/R, R*), in which two *R-r:std* alleles were transmitted paternally on the *B•10 (R)* chromosome, produced mottled kernels similar to (*r, r/R*) (normal) kernels. This result indicated that mottling was not due to a dosage effect but was caused by a parental difference in expression of *R-r:std*. Other alleles produce non-imprinted seed color such as the *Sc* allele (*self-color*) (shown in Figure 1C).

Similar to mammalian genomic imprinting, *r1* imprinting phenotypes reflect a difference in methylation of *r1* alleles in which maternally transmitted *R-r:std* is less methylated than paternally transmitted *R-r:std* (M. Alleman, unpublished information). An important issue regarding the process of *r1* imprinting is determining the default state of the allele. In other words, is maternal *R-r:std* de-methylated or does paternal *R-r:std* undergo *de novo* methylation? Southern gel blot analysis of DNA from young kernels using methylation-sensitive restriction enzymes was used to compare maternally and paternally transmitted *R-r:std* in the endosperm and embryo. The effect of imprinting is on the maternally transmitted allele, which undergoes a specific de-methylation in the endosperm relative to the paternally transmitted allele or the same allele in embryonic tissue. Although the *R-r:std* allele is expressed exclusively in the aleurone layer of the maize endosperm, the change in methylation is seen throughout the endosperm (M. Alleman, unpublished information).

Because the egg nucleus and one of the two maternal endosperm nuclei are the products of a single mitotic cell division during gametophyte development, the methylation changes that occur during *r1* locus imprinting must occur after central cell formation. *R-r:std* would undergo a change from relatively methylated to relatively under-methylated in the central cell of the female gametophyte. Alternatively, an imprint could be placed on *R-r:std* in the central cell and de-methylation could occur after fertilization during endosperm development. In this system, there is no reason to invoke the existence of an 'imprint' to differentiate these alleles at later stages of development. The requirement for an imprint is based on the necessity that an allele is marked during gametogenesis, that the mark is interpreted later, and, in the germ line,

the mark can be removed. Because the endosperm is a terminally differentiated tissue, an imprinting mark is unnecessary. If de-methylation of *R-r:std* occurs in the central cell of the embryo sac, the methylation state of maternal and paternal alleles can be propagated after fertilization using maintenance methylation functions.

#### *Imprinting and paramutation of the R-r:std allele*

The degree to which paternal *R-r:std* is expressed is dependent on the epigenetic state of the particular allele (Brink, 1958). Epigenetic states of *rl* alleles also can involve the process of paramutation (Chandler *et al.*, this issue). Methylation and the phenotypic difference between maternally and paternally transmitted *R-r:std* is increased during paramutation. Paramutation is defined as homology-dependent silencing of one allele by another in specific heterozygotes (Chandler *et al.*, this issue). Alleles that induce silencing, such as *R-stippled* (*R-st*), are called paramutagenic; those that are sensitive to silencing, such as *R-r:std*, are paramutable. The altered form of *R-r:std* is called paramutant and is denoted *R-r:std'*. Paramutation results in a progressive increase in cytosine methylation of paramutable alleles with a decrease in anthocyanin production in the maize aleurone (Kermicle and Alleman, 1990; Walker, 1998).

The relationship between paramutation and imprinting is of interest here. Paramutation results in a substantial increase in the phenotypic difference between maternally and paternally transmitted alleles (imprinting). Nonetheless, paramutation and imprinting may be considered separate epigenetic phenomena. Some paramutable alleles show imprinting only when in the paramutant form but not in the native form (Kermicle, 1978). In addition, imprinting occurs during gametophyte development while paramutation occurs in the sporophyte (Brink, 1958). Paramutation is generally viewed as an endogenous form of homology-dependent gene silencing (Matzke *et al.* 1996). *R-r:std* epialleles are methylated throughout the plant, regardless of whether they are expressed in a particular tissue type. Maternal transmission of *R-r:std* or *R-r:std'* during gametogenesis will result in specific de-methylation of *R-r:std* DNA in the endosperm of the kernel and in solidly pigmented kernels (Figure 1A and B). Thus, imprinting and paramutation are related only in that they affect the kernel phenotype of the same allele, but result in methylation changes in the opposite direction.

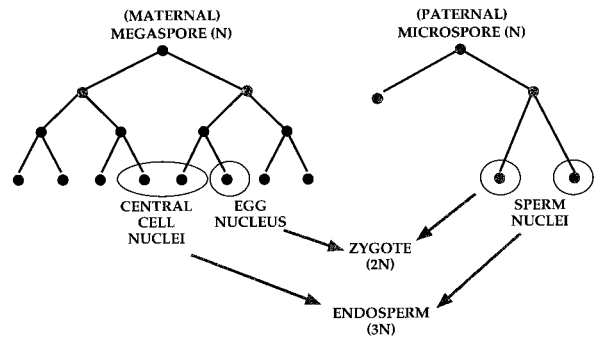


Figure 2. Gametophyte development in angiosperm plants. Flowering plants undergo double fertilization. Two sperm are produced from a single product of meiosis during microsporogenesis; one fertilizes the egg mother cell to produce the diploid, embryonic sporophyte; the other fertilizes the diploid central cell to produce a triploid endosperm. Gametophyte development begins with a single product of meiosis and ends with two sperm and a generative nucleus (microspore development) or eight mitotic products including the egg and central cell (megaspore development).

#### *Imprinting and paramutation at rl: structural considerations*

'Imprinting' versus 'non-imprinting' represents a major dichotomy among *rl* alleles, based on allele structure and probably having a phylogenetic basis. Similarly, paramutagenic and paramutable alleles represent distinct structural classes of *rl* haplotypes. The structural complexity of the *rl* locus is presumed to be the source of both imprinting and participation in paramutation. Strongly paramutagenic alleles always are composed of multiple genes in direct orientation. Reducing gene number in *R-st* causes a decrease in paramutagenicity to nearly or completely non-paramutagenic for single-gene *rl* alleles (Eggleston *et al.*, 1995; Kermicle *et al.*, 1995). The structural features of paramutation are consistent with homology-dependent gene silencing found in transgenic plants in which increases in gene copy number correlate with decreased gene expression and increased cytosine methylation (Matzke *et al.*, 1994).

In contrast with paramutagenic alleles, known paramutable alleles contain an inverted repeat of two seed pigmentation genes with a centrally located promoter called *Sigma* (Robbins *et al.*, 1991; Walker *et al.*, 1995; R. Okagaki and J. Kermicle, GenBank accession number U93178). The structure of *Sigma* varies in each of three allele types (Walker *et al.*, 1995; M. Alleman, unpublished information). One type of *Sigma* structure, from *R-r:std*, is shown in Figure 2. This structure differs somewhat from the *R-r:std* *Sigma* structure proposed by Walker *et al.* (1995).

From the diagrammed structure, it is obvious that several tiers of repeated sequences are present. These repeats include the *S1* and *S2* transcribed regions, small (12 to 16 bp) repeats originating from the *Doppia* transposable element, and proposed promoter element repeats (Walker *et al.*, 1995).

An inverted-repeat structure is important for epigenetic silencing of the PAI locus in *Arabidopsis*. Inverted repeats may direct DNA-DNA interactions resulting in changes in cytosine methylation that are transferred between the repeats and to ectopic repeats (Luff *et al.*, 1999; Bender, 1998). It is also interesting that some mammalian imprinted genes have overlapping transcripts that are divergently transcribed, although not from a common promoter. Often, one of the genes, such as *H19*, is not translated (Tilghman, 1999). In addition, mammalian DMRs occur adjacent to blocks of different types of short directly repeated sequences (Neumann *et al.*, 1995). Although a difficult hypothesis to test experimentally, the presence of repeated sequences is a common feature of epigenetically silenced and imprinted genes.

The ability to undergo imprinting is a property of specific *r1* alleles, not a characteristic of the *r1* locus *per se*. With the exception of the mouse versus *Peromyscus Rasgrf1* genes, imprinting affects all alleles for mammalian genes. This dichotomy between different *r1* alleles is likely based in gene structure, rendering *R-r:std* both paramutable and able to undergo imprinting. Of note, rare *cis* mutations involving the imprinting of *R-r:std* result from deletions that are located adjacent to *Sigma* (M. Alleman, unpublished information). None of these mutations affects paramutation. It is possible that some structural changes disrupt a process that is necessary for imprinting but not paramutation. For example, paramutation may require pairing between regions of *R-st* and *R-r:std* and involve regions in the same orientation, but imprinting may require pairing between the inverted segments in *S1* and *S2*.

#### *mdr genes and modification of imprinting*

The role of methylation and imprinting in the development of the maize endosperm can be studied through genes that regulate these processes. *mdr1* was the first gene found to affect imprinting directly (Kermicle, 1978). *mdr1* (*maternal de-repression of r1*) is located on chromosome 4 and controls *r1* locus imprinting in the endosperm (Kermicle, 1978). The *imp1* gene of mouse may be another example of an imprinting

control gene (Forejt and Gregorová, 1992). In combination with the mutant *mdr1-r* (*reference*) allele, maternally as well as paternally transmitted *R-r:std* or *R-r:std'* alleles specify a mottled phenotype. *mdr1-r* also shows a parent-of-origin effect in the female gametophyte. When transmitted through the pollen, the wild-type *Mdr1-w22* allele does not correct the *mdr1-r* phenotype (Kermicle, 1978). This would suggest that either *Mdr1* itself is imprinted or that it acts during the gametophyte stage of development. Figure 1D shows the *R-r:std* phenotype in the presence of a maternally inherited *mdr1-r* mutant.

A prediction regarding the interaction between *mdr1* and *R-r:std* would be that loss of *Mdr1* function blocks de-methylation of *R-r:std* in the endosperm. This appears not to be entirely correct. Preliminary data indicate that *r1* DNA from the maternally inherited genotype *R-r:std*, *mdr1-r* is methylated to an intermediate degree relative to maternally inherited *R-r:std*, *Mdr1-w22* (unmethylated), or paternally inherited *R-r:std* (highly methylated) (M. Alleman, unpublished information). This suggests that multiple *mdr* genes are present in the maize genome.

#### *Imprinting of multi-copy genes in the maize endosperm*

The general assumption is that, for most genes, both maternally and paternally transmitted alleles are expressed at equivalent levels, and that imprinting does not occur widely. We suggest a link between imprinting and development of the angiosperm endosperm. Mutations in over 300 maize genes have visible endosperm phenotypes upon transmission by both parents (Neuffer *et al.*, 1986). Nonetheless, maize kernels are matroclinous; the progeny of kernels of crosses between diverse races are structurally (shape and size) like the maternal parent. For example, a cross of maize (maternal) by teosinte (paternal) produces maize-like kernels. Only the progeny of that cross will express teosinte-like endosperm traits. Thus, it appears that genes governing seed size and shape are expressed from maternal alleles (Schwartz, 1965; Kermicle, 1978). Imprinting in plants might represent one level of control of gene expression used in the endosperm to maintain maternal control of kernel growth and development, in a tissue that is structurally dependent on a maternal organ, the cob.

On the other hand, mutations of some genes have strong maternal-specific phenotypes in the endosperm. These include *floury-1* and pH 7.5 *esterase* (Schwartz,

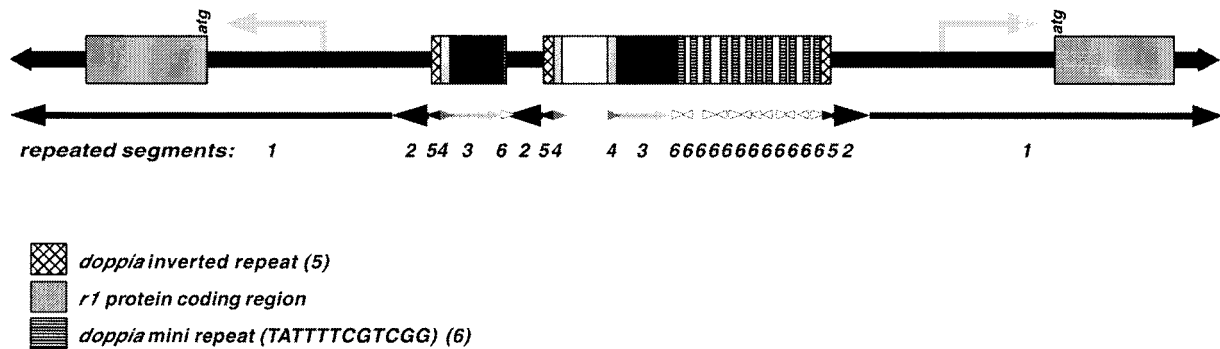


Figure 3. The complex structure of the *Sigma* region of *R-r:std*. The upstream region of the *S* subcomplex of the *R-r:std* allele is composed of multiple inverted and direct repeats. Unique structures are shown using shaded boxes. The arrows represent individual repeated elements which are denoted by the numbers 1–6: 1 and 2 are *S1* or *S2* coding regions; 3 is the *doppia* inverted repeat; 4 and 5 are segments that also occur in the 5'-flanking region of the *r1* genes *P* and *Sc* (Y. Li and M. Alleman, unpublished information); 6 is the *doppia* mini repeat (Walker *et al.*, 1995). The *S1* and *S2* coding regions extend to the right and left of the figure for ca. 7 kb. *Sigma* includes all sequence between the two *S* gene-coding regions. The start of translation is consistent with that of the *Lc* gene and is based on an *S2* gene cDNA sequence (Perrot and Cone, 1989; Ludwig *et al.*, 1989). The positions of the origin of transcription for the *S* genes are unknown but are presumed to be between *Sigma* and the start of translation (as marked on the diagram) based on a published cDNA sequence (Perrot and Cone, 1989). The *doppia* region has been described by Walker and colleagues and is the remnant of a putative transposable element (Walker *et al.*, 1995). The segment marked inverted repeat is the presumed terminal sequence of the *doppia* transposable element.

1965), *floury-2* (Di Fonzo *et al.*, 1980), *dzr1* (*delta zein regulator-1*) (Chauduri and Messing, 1994), and *Dap* (*defective aleurone pigmentation*) (Gavazzi *et al.*, 1997). Besides *esterase*, these genes are involved in endosperm functions or have a direct effect on endosperm development. The *dzr1* gene is a post-transcriptional regulator of the 10 kDa *zein* storage-protein gene (Chauduri and Messing, 1994). For this gene, the evidence for imprinting is based on the behavior of heteroallelic combinations of variant alleles from inbred maize lines. It may be significant that *dzr1* maps to a chromosome arm (4S) that exhibits parent-of-origin effects on endosperm development characterized by a small-kernel phenotype.

#### Parent-of-origin methylation effects and imprinting of gene expression

Parent-of-origin-specific methylation patterns, although not proven to involve imprinting of gene expression, are of considerable interest because of the strong association of differential methylation with known cases of genomic imprinting. In maize and wheat, differential methylation of several gene families occurs in the endosperm and is associated with several cases of imprinting (Finnegan *et al.*, 1993). The  *$\alpha$ -tubulin* gene family, which is expressed in all plant tissues, shows differential methylation of some family members in the plant, embryo, and endosperm. Polymorphisms between  *$\alpha$ -tubulin* genes from diverse

inbred maize lines were used to show that specific demethylation occurs for some maternally inherited alleles in the endosperm and was correlated with increased gene expression (Lund *et al.*, 1995b). Similarly, some subfamilies of *zein* genes are under-methylated and show increased transcription in the endosperm relative to the plant or embryo. Under-methylation affects the maternal copies exclusively (Lund *et al.*, 1995a; Bianchi and Viotti, 1988).

Thus, some alleles at the *r1* locus, and  *$\alpha$ -tubulin* and *zein* gene subfamilies show analogous parent-of-origin patterns of methylation and expression. The male gametophyte, the embryo, and the plant comprise the native state of these genes, a relatively methylated, and potentially under-expressed, condition. In each system, the maternal alleles are specifically demethylated in the endosperm, and this state is correlated with increased gene expression. Similarly, in each case, only certain alleles are affected. Thus, for these genes, imprinting is an *allele*-specific, not a *locus*-specific, phenomenon as in mammals.

#### The Arabidopsis MEDEA gene

The Arabidopsis *MEDEA* gene challenges the view that imprinting occurs solely in the endosperm, as observed for the maize *R-r:std* allele. Imprinting of *MEAI* occurs in both the embryo and the endosperm, as indicated by developmental profiles of RNA expression. The *meal-1* mutant was isolated by Grossniklaus

*et al.* (1998) in *Ds* transposable element mutagenesis. They noted the significance of this mutation, and named it *MEDEA* for the Greek legend of Medea, who was blamed for killing her children. *meal-1* results in aborted seed development upon maternal transmission, and, similar to *mdr1-r* of maize, wild-type copies of paternally transmitted *MEAI* do not rescue the mutant phenotype. The *meal-1* mutant phenotype is first apparent as a delay in morphogenesis at the globular stage in *Arabidopsis* embryos, resulting in larger heart- and torpedo-stage embryos. The endosperm also appears abnormal and shows delayed nuclear division and cellularization in the presence of maternally transmitted *meal* mutations. *meal* seeds abort during desiccation. Thus, both endosperm and embryo undergo abnormal development, though discrepancies exist in the precise description of the tissue-specific phenotypes among various independently isolated alleles (*meal-2* or *fis1*, Chaudhury *et al.*, 1997; Luo *et al.*, 1999; *meal-1*, Grossniklaus *et al.*, 1998; *meal-3* or *f644*, Kiyosue *et al.*, 1999; *meal-4* or *emb173*, Castle *et al.*, 1993).

*MEAI* encodes a SET-domain protein homologous to members of the *polycomb* family of genes in *Drosophila* (Grossniklaus *et al.*, 1998). This class of genes is involved in the maintenance of chromatin structure, presumably regulating access of transcription factors to *cis* binding sites in regulated genes. The *MEAI* transcript appears in *Arabidopsis* during megagametophyte development and is present during seed development as well as at low levels in the sporophyte (Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999). Because of the fertilization-independent phenotype of *f644* (*meal-3*), *MEAI* is presumed to encode a product whose maternal function is to repress endosperm development, causing embryo abortion by disrupting endosperm/embryo interactions during early development (Kiyosue *et al.*, 1999). A role in sporophyte development is also indicated by the expression of *MEAI* in the embryo and plant. Because of the slightly reduced penetrance of maternally inherited *meal* alleles, it is proposed that other related SET-domain proteins, such as CURLY LEAF, may compensate for loss-of-function *meal* alleles (Kiyosue *et al.*, 1999).

A variety of explanations are possible for the maternal-specific phenotype of the *meal* mutations including (1) haplo-insufficiency of *meal* product, (2) gametophytic tissue specificity, and (3) genomic imprinting. Haplo-insufficiency and gametophytic tissue specificity can be ruled out based on expression of the *MEAI* product during post-fertilization periods in the

endosperm and embryo. Support for imprinting of the *MEAI* gene comes from studies on the pattern of RNA accumulation from maternally and paternally derived alleles during *Arabidopsis* development (Kinoshita *et al.*, 1999; Vielle-Calzada *et al.*, 1999). *MEAI* is imprinted in the developing endosperm and in the embryo, both showing maternal expression very early in development (Vielle-Calzada *et al.*, 1999). This makes *MEAI* the first example of single-gene imprinting in plants that is not exclusively endosperm-specific.

Although maternally imprinted, paternal expression of *MEAI* occurs in the embryo by the torpedo stage and in the endosperm for specific ecotypes (Kinoshita *et al.*, 1999). This paternal allele expression of *MEAI* is apparently not sufficient to correct the *meal* mutant phenotype of seed abortion. Interestingly, imprinting of *MEAI* is dependent on *DDM1* (*decreased DNA methylation*), a gene with effects on long-term maintenance of methylation states in *Arabidopsis* and homology to SWI2/SNF2-like chromatin remodeling proteins found in yeast (Vongs *et al.*, 1993). Paternal transmission of *ddm1* mutant and *MEAI* wild-type alleles allows expression of *MEAI* paternal alleles (Vielle-Calzada *et al.*, 1999). This paternal expression of *MEAI* rescues seed viability, although the seeds are larger than wild type and share other abnormalities of maternally inherited *meal-1* seeds. The interaction of *MEAI* and *DDM1* highlights the involvement of methylation and/or chromatin structure in the imprinting process in plants.

## Perspectives

### *Imprinting: important and unimportant genes*

Translocation-based studies used to map parent-of-origin effects to chromosomal regions in the mouse have been linked, in many cases, to molecularly characterized imprinted genes at these chromosomal locations. The functions of many of these genes are crucial for early development. On the other hand, in maize, sub-chromosomal regions identified as having a small-kernel effect do not contain molecularly identified imprinted genes. Those imprinted genes that have been characterized at a molecular level include storage protein genes and alleles of the *r1* locus. Reduced seed size from chromosomal imbalances cannot be attributed to imprinting effects at these loci. Thus, as seen in other organisms such as *Drosophila*, imprinted genes that are currently known are not essential for development. Imprinted loci that result in the

small-kernel effect may be important for endosperm development but, clearly, not essential in the embryo. It is possible that most genes needed for endosperm development are functionally duplicate, and at least partially complement each other. The *MEDEA* gene of *Arabidopsis* has become the only 'crucial' gene to have an imprinting effect early in plant development (Kinoshita *et al.*, 1999; Vielle-Calzada *et al.*, 1999).

#### *Evolutionary consequences of imprinting*

On the surface, it appears that imprinting imposes an evolutionary disadvantage. By silencing one allele in a diploid, imprinting renders a heterozygous locus as a functional hemizygote and may, thus, place individuals at a disadvantage for survival. To account for the evolution of imprinting, several explanations have been advanced (Hurst and McVean, 1998).

A theoretical consideration of imprinting dictates that a strong selective advantage, such as is provided by heterozygote advantage (heterosis), is necessary for these systems to evolve (Spencer and Williams, 1997). This is consistent with Kermicle's epihybridity model which suggests that imprinting in maize occurs in the endosperm to produce a tissue-specific type of hybrid vigor (Kermicle and Alleman, 1990). This line of reasoning is also consistent with the 'parental conflict' hypothesis for the evolution of imprinting (Haig and Westoby, 1989; Moore and Haig, 1991). Conflict or epihybridity explains why imprinting of developmentally essential genes has only been observed in eutherian mammals and angiosperm plants. In both types of organisms, embryos can compete for maternal resources – in eutherian mammals through the placenta, and in plants through the endosperm. Because many mammals and angiosperms are not monogamous, the 'parental conflict' hypothesis predicts that genes from the male parent will be imprinted to enhance embryo growth at the expense of maternal resources dedicated to other offspring, and that maternal genes will be imprinted to limit the growth of any particular embryo. Several of the first imprinted genes to be discovered in mammals were indeed important for embryonic growth, as predicted by the hypothesis. More recently, however, imprinted genes in mammals have been identified whose function is not easily reconciled with the 'parental conflict' model (Hurst and McVean, 1998).

#### *Imprinting in plants: some final thoughts*

Genomic imprinting occurs in a variety of organisms. In both plants and mammals, there is a requirement

for the genomes of both parents for successful development. In plants, if imprinting is a means to maintain developmental control of developmentally essential genes by the mother, why then are alleles of non-essential genes, such as *R-r:std* and other *r1* alleles, under imprinting control?

A connection can be made with a general model of epigenetic silencing in higher organisms. This model recognizes that all organisms are capable of silencing, and that certain DNA sequences are targets for the type of silencing that results in genomic imprinting. These include direct repeat sequences, inverted repeats, and other DNA motifs such as DMRs, represented in the known imprinted genes. Experimental evidence indicates that, by creating transgenic organisms, we produce the potential for genomic imprinting in the form of parent-of-origin-specific cytosine methylation (zebrafish) or chromatin structural differences (*Drosophila*). What is the connection between imprinting of endogenous genes and transgenes? Perhaps the potential for genomic imprinting exists in all organisms that are able to utilize systems of epigenetic silencing. Plants and mammals have exploited this potential. Genomic imprinting can be used as an important mechanism in the control of the expression of growth or development-regulating genes such as *MEDEA* or *Igf2r*. Cereal plants such as maize have another goal for imprinting, to retain maternal control of seed form. Only further analysis, using genetic and molecular approaches, will reveal to what extent common mechanisms unite imprinting, gene silencing, and other epigenetic phenomena among diverse species.

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