Imprinting in the endosperm: a possible role in preventing wide hybridization

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Reproductive isolation is considered to play a key part in evolution, and plants and animals have developed a range of strategies that minimize gene flow between species. In plants, these strategies involve either pre-zygotic barriers, such as differences in floral structure and pollen-stigma recognition, or post-zygotic barriers, which are less well understood and affect aspects of seed development ranging from fertilization to maturation.

In most angiosperms, a double fertilization event gives rise to a zygote and the endosperm: a triploid tissue with an unequal parental genomic contribution, which, like the placenta of mammals, provides reserves to the developing embryo. Interestingly, many aspects of endosperm development, again like the placenta, are regulated by a range of epigenetic mechanisms that are globally termed imprinting. Imprinted genes are characterized by their uniparental expression, the other parental allele being silenced. Normal development of the endosperm thus requires a highly specific balance of gene expression, from either the maternal or paternal genomes. Any alteration of this balance resulting from changes in allelic copy number, sequence or epigenetic imprints can cause endosperm failure and eventual seed abortion. In its widest sense, the endosperm thus serves as an accurate 'sensor' of compatibility between parents. A first step in understanding this important, yet complex system must clearly be the isolation and characterization of as wide a range as possible of imprinted genes.

Keywords: silencing; epigenetic; imprinting; endosperm; hybridization

1. INTRODUCTION

Since the discovery of double fertilization (Nawaschin 1898; Guignard 1899) and the existence of the endosperm just over a century ago, the role of this structure in angiosperm reproduction has remained puzzling. Certainly, development of the endosperm is necessary for the correct development of the embryo and, as early as 1942, Cooper & Brink (1942) proposed a role for endosperm failure in reproductive isolation and angiosperm speciation. The genetic basis of endosperm failure remains unclear, although gene dosage effects (Birchler 1993) and imprinting of regulatory genes (Haig & Westoby 1991) have been proposed as contributory factors.

Imprinting is a mitotically stable epigenetic modification of parental alleles that leads to the differential expression of genes in a parent-of-origin manner. Imprinting therefore renders parental genomes non-equivalent, and for this reason the 'correct' contributions of maternal and paternal genomes are essential for post-fertilization development (Spielman *et al.* 2001; Surani 2001). Interestingly, whereas mammalian embryo development requires the participation of both parental genomes, the same does not hold for all angiosperms, where maternally

or paternally derived haploid embryos can produce viable seedlings—as can maternal diploid embryos of apomictic angiosperms (Grimanelli *et al.* 2001). Imprinting thus appears to play a far more significant part in endosperm development (Haig & Westoby 1991) than in embryogenesis. Haig & Westoby (1991) also proposed that the unusual triploid composition of the endosperm possibly led to the selection of parentally imprinted genes that play an important part in the function of the endosperm in reproductive strategies.

2. GENETIC EVIDENCE FOR IMPRINTING IN THE ENDOSPERM

Evidence that imprinting is, in part, responsible for endosperm failure is largely derived from studies of intra-specific crosses between different ploidy levels, and crosses between related species. Reciprocal crosses performed between diploid and tetraploid individuals in a range of species have been reported to result in abnormal endosperm development (Brink & Cooper 1947; Redei 1964; Johnston *et al.* 1980). Likewise, interploidy crosses carried out in maize using *indeterminate gametophyte (ig)*, a mutant that generates embryo sacs with a variable number of cells and nuclei, indicate that deviations from the normal two maternal to one paternal (2m : 1p) genomic balance in the endosperm resulted in abnormal development, perhaps owing to an imbalance of imprinted sequences within the endosperm (Lin 1984). Although most studies suggest

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Figure 1. AMD strategy for the identification of imprinted genes in maize endosperm. (a) Schematic diagram of band patterns expected after RT-PCR amplification and separation by electrophoresis, showing cDNA polymorphisms between endosperm mRNA samples isolated from two selfed and reciprocally crossed maize inbred lines, A and B. Monomorphic cDNAs are represented by the same sized band located in all four endosperm samples. Among polymorphic cDNAs, biallelic expression is represented by the presence of both parental bands in reciprocal endosperm samples (AB and BA); dosage expression gives a similar pattern to biallelic expression only with a stronger band signal for the maternal allele in reciprocal endosperm samples; monoallelic expression is represented by the absence of either a maternal or paternal band in reciprocal endosperm samples and are good candidates for genes subjected to a paternal and maternal parent-of-origin expression pattern, respectively. (b) Autoradiograph showing a typical AMD gel. Each lane displays different cDNAs from selfed and reciprocally crossed maize inbred lines (as above), amplified by RT-PCR using a range of random primer combinations. Note the presence of a paternal parent-of-origin pattern of expression (arrows).

that the 2m : 1p ratio is essential for endosperm development, there are some exceptions. Lin (1984) showed that 3m : 1p endosperms developed, whereas 6m : 2p endosperms did not. In another approach, Rhoades & Dempsey (1966) generated tetraploid plants from *elongate* mutant lines, which produce unreduced gametes. When crossed with diploid, tetraploid and hexaploid males, endosperms were formed with a diverse number of ploidies ranging from 3x to 11x. The only endosperms that developed normally were those of diploid and tetraploid males, although a few kernels were obtained from crosses with a hexaploid male. Interestingly, self-pollination of hexaploid and octoploid maize results in high female sterility (Randolph 1932). One possible explanation is that high ploidy in the endosperm might have caused other developmental abnormalities, separate from those resulting from deviation from the 2m : 1p ratio that affect lower endosperm ploidies (Birchler 1993). More convincing evidence of imprinting operating in the endosperm came from chromosomal translocation studies in maize. Lin (1982) found that the absence of a paternally inherited chromosome arm in the endosperm resulted in reduced seed size, and that extra female copies of this chromosome arm failed to compensate for the absence of the paternally transmitted copy. Conversely, evidence of a dosage mechanism operating in the endosperm was provided by Birchler & Hart (1987), who demonstrated that extra maternal copies of chromosome arms *other* than the arm absent from the paternal genome also accentuated a reduction in kernel size.

Taken together, the evidence found to date strongly suggests that dosage effect and imprinting of different loci are the main contenders for regulating endosperm development in maize, and most probably in other angiosperms.

3. AN OVERVIEW OF IMPRINTED GENES IN PLANTS

The first plant gene that was demonstrated to be imprinted was the endosperm-expressed *r1* gene of maize (Kermicle 1970, 1978). In a series of outstanding studies, Kermicle not only identified imprinting in this gene, but also genetically demonstrated the existence of a regulatory element, the maternal derepressor of *r* (MDR) (Kermicle 1978).

Almost 20 years later, additional examples of imprinting in plants were identified including genes encoding zeins, tubulins and a posttranscriptional regulator of zeins (*dzt1*) (Chaudhuri & Messing 1994; Lund *et al.* 1995a,b). A common feature of these imprinted genes is that their expression is endosperm-specific, they are members of multi-copy gene families and the imprinting seems to be allele-specific only in some inbred lines.

Recently, locus-specific imprinting has been identified in genes of the FIS class in *Arabidopsis*. MEDEA was identified as a lethal gametophytic maternal effect mutant (*mea*) and encodes an enhancer of zeste (E[Z]) polycomb protein (Grossniklaus *et al.* 1998; Kinoshita *et al.* 1999). Later studies suggest that MEDEA expression is imprinted in both embryo and endosperm (Vielle-Calzada *et al.* 1999). The other two genes of the FIS class (*FIE*, *FIS2*) characterized in *Arabidopsis* are exclusively maternally

Table 1. (a) Sequences revealed by AMD screening of 6 and 15 DAP endosperms, and (b) putative functions of some characterized sequences.

(a)				
samples	paternal	maternal	dosage	percentage
6 DAP	1	15	65	2–5
15 DAP	0	31	125	3–6
(b)				
putative function	paternal	maternal	dosage	total
structural	0	6	4	10
metabolic	1	2	10	13
transcription	0	2	1	3
chromatin	0	1	0	1

expressed in the endosperm, and belong to the polycomb group of proteins (Luo *et al.* 1999; Yadegari *et al.* 2000). *FIE* seemingly interacts with *MEDEA*, and among other roles, may be involved in the establishment of the anterior–posterior axis in early endosperm development (Spillane *et al.* 2000; Sorensen *et al.* 2001). Surprisingly, different mechanisms may regulate the parent-of-origin expression seen in *mea* and *fie* mutants, since plants with reduced global methylation used as pollen donors can rescue *fis2* and *mea* but not *fie* (Luo *et al.* 2000; Vinkenoog *et al.* 2000). Similarly, pollen from a mutant that affects chromatin conformation and DNA methylation (*ddm1*) can rescue *mea* but not *fie* (Yadegari *et al.* 2000). Most recently, *DEMETER* (*DME*), a DNA glycosylase, has been demonstrated to be required for the specific activation of *MEA* in the megagametophyte (Choi *et al.* 2002).

A delay of paternal genome activation in the *Arabidopsis* embryo and endosperm has been reported (Vielle-Calzada *et al.* 2000), and it has been proposed that this genome-wide silencing of paternally inherited genes may affect the vast majority of loci—excluding those with a major role in early seed development (Baroux *et al.* 2002). However, *in vitro* data from maize suggest that transcription of paternally transmitted transgenes occurs in zygotes shortly after fertilization, pointing to activity of the male genome, at least in maize, almost immediately after karyogamy (Scholten *et al.* 2002). Whether the entire paternal genome, or just a fraction of it, is available for early zygotic and endosperm development thus remains unresolved.

These findings suggest that imprinting in plants is a complex process common, but perhaps not exclusive, to the endosperm. Further, it appears that imprinting operates via multiple mechanisms: allele-specific (most sequences described in maize to date), gene-specific (*MEDEA*, *FIS* and *FIE* in *Arabidopsis*), and genome-wide (the reported paternal genome silencing in *Arabidopsis*). The isolation and characterization of further imprinted genes in an extended range of species should assist our understanding of the operation, and the evolutionary origin of this intriguing and important process.

4. GENOME-WIDE SCREENING FOR IMPRINTED GENES IN PLANTS

Mutant screens have been of great value in isolating imprinted genes in *Arabidopsis* (Baroux *et al.* 2002) but

have proven less useful in other species with a more complex genomic organization. To identify novel imprinted sequences in the maize endosperm we have modified a molecular screen termed AMD, originally developed to isolate imprinted sequences in mouse (Hagiwara *et al.* 1997). In principle, AMD is based on a differential display PCR technique that allows visualization of multiple ‘transcripts’—in reality 3′-ends of arbitrary cDNAs derived from tissue- or stage-specific mRNAs—regions rich in polymorphisms among maize inbred lines (Bhatramakki *et al.* 2002). AMD can therefore reveal polymorphic alleles and their parental expression levels in endosperm samples isolated from self-pollinated and reciprocally crossed parental inbred lines (figure 1). Using a combination of arbitrary and anchor primers, it is possible to generate a unique ‘fingerprint’ of polymorphic bands representing four expression categories: biallelic, dosage-dependent, exclusively paternal and exclusively maternal (figure 1a). In a comparative analysis of two developmental stages (6 and 15 DAP), between 2% and 6% of the amplified polymorphic sequences exhibited parent-of-origin expression. A large proportion of these sequences belonged to structural and biochemical gene families, whereas a smaller number were transcriptional regulators and genes involved in chromatin architecture (table 1). Significantly, most of these sequences were expressed in a dosage-dependent manner with only a small minority of sequences showing exclusively maternal or paternal expression patterns (table 1). Further molecular characterization of the one clearly paternally imprinted sequence detected, *PEG1*, showed that its parent-of-origin expression pattern is restricted to the endosperm—the gene being biallelically expressed in the embryo (figure 2a). This is the first report, to our knowledge, of a gene showing a paternal parent-of-origin expression pattern in plants. We also identified a small gene family, homologous to the *Arabidopsis FIE* gene (Springer *et al.* 2002), which showed an exclusively maternal pattern of expression. Of this family, *FIE101* is only expressed in the endosperm and imprinted throughout endosperm development (figure 2a), whereas *FIE102* is biallelically expressed in the embryo, but maternally expressed only at early stages of endosperm development (figure 2a). The differences found in expression patterns and parent-of-origin effects strongly indicate that the maize *FIE*-like sequences may have evolved to assume divergent roles during maize evolution (Danilevskaya *et al.* 2003).

Figure 2. Several types of parent-of-origin expression patterns operate in maize endosperm. (a) Embryo and endosperm (polymorphic) mRNA accumulation of parental inherited alleles after selfing and reciprocally crossing four maize inbred lines (where A is W22; B, Tx303; C, Mo17; and D, B73). *PEG1* shows a paternal parent-of-origin pattern of expression in endosperm and biallelic expression in embryo. The endosperm-specific gene, *FIE101*, shows a maternal pattern of expression throughout endosperm development, whereas *FIE102* shows a maternal parent-of-origin pattern of expression only at early stages of endosperm development and is biallelically expressed in embryo. (b) Early endosperm (6 DAP) mRNA expression of parental alleles after selfed and reciprocally crossed diploid and tetraploid maize inbred lines. Glutathione synthase expression was used as a control for biallelic expression.

Our study clearly demonstrates how a genomic screening approach can be effective in identifying maize endosperm sequences expressed in a parent-of-origin pattern. While the sequences we have characterized to date are unambiguously imprinted in the maize endosperm, much further study is required before a clear understanding can be gained of the mechanisms by which imprinting operates during early embryo and endosperm development. Significantly, the parent-of-origin expression patterns of *PEG1*, *FIE101* and *FIE102* in the endosperm are not affected by interploidy crosses (figure 2b), which suggests that the imprinting mechanism continues to function in the endosperm despite alterations to the maternal to paternal genomic ratios.

5. FAILURE OF EARLY ENDOSPERM DEVELOPMENT IN INTERPLOIDY CROSSES AND HYBRIDS

Cooper & Brink (1942) were among the first to ascribe a major role to the endosperm in the failure of seed production after interspecific hybridization. Histological studies on hybrid maize showed abnormalities at very early stages of endosperm development ranging from defects at the chalazal pole (Cooper & Brink 1940), to abnormal free nuclear division rates (Brink & Cooper 1940). Interploidy crosses in monocot species also exhibit altered rates of endosperm cellularization (Kihara & Nishiyama 1932; Wakakuwa 1934; Cooper 1951; Håkanson 1953), while similar crosses in dicots reveal cellularization to occur early with the production of fewer cells in maternal excess.

Dicot endosperms with excess paternal genomes exhibit delayed cellularization and elevated rates of cytokinesis (Valentine 1955; Nishiyama & Inomata 1966; Scott *et al.* 1998).

Evidence is thus accumulating that failure of the polygonum-type endosperm in interspecific and interploidy hybrids results from abnormal development at, or about, the free nuclear stage, since hybridizations involving species that do not undergo a free nuclear stage of development do not respond in this way (Sansome *et al.* 1942; Cooper & Brink 1945).

While many of the regulators of embryo and endosperm development have now been identified (comprehensively reviewed in Jurgens (2001) and Olsen (2001)), the molecular basis of these abnormalities in young endosperm development during the free nuclear stage is not well understood. Previous studies have however, revealed that the BETL cells develop abnormally in endosperms resulting from diploid by tetraploid crosses (Charlton *et al.* 1995). To explore these early developmental changes in a structure that is largely inaccessible to the normal techniques of molecular analysis, we have employed a maize transgenic line carrying an endosperm-specific reporter construct that permits the identification of BETL cells in the developing endosperm (figure 3a-c). Preliminary analysis has shown dramatic changes in the distribution pattern of this marker following interploidy crosses, ranging from limited expression in the BETL, to a few aberrant cells localized in the apical region of the endosperm (figure 3b,c). Further, *in situ* hybridization analysis of an early transcriptional activator of BETL-specific

Figure 3. Abnormal localization of BETL transcripts in interploidy maize kernels. β -glucuronidase localization at 10 DAP in balanced (2m : 1p) triploid endosperms is confined to basal transfer cells (a), restricted to a small group of transfer cells in the maternal excess (4m : 1p) interploidy endosperms (b), and limited to a few cells in the apex in a paternal excess (2m : 2p) interploidy endosperm (c). Distribution of ZmMRP transcript during early endosperm development was found in the basal portion of maternal excess interploidy endosperms (d), and in few basal and apical cells of the early, cellularizing, paternal excess interploidy endosperm (e). Scale bars, 100 μ m.

genes, *MRP* (Gomez *et al.* 2002), in interploidy endosperms undergoing cellularization showed abnormal patterns of expression (figure 3d,e).

Interestingly, specification of BETL in cereals is held to occur in the free nuclear or coenocytic endosperm (Olsen 2001), and mRNAs for *MRP* have been localized in the basal portion of the coenocyte a few hours after fertilization (Gomez *et al.* 2002). Taken together with our data, these observations suggest that following interploidy crosses, developmental regulators become aberrantly localized within the coenocytic endosperm, leading to an inappropriate distribution of cells with BETL identity in the cellular endosperm.

The parental balance of genomes in these interploidy crosses is also clearly of importance since expression of BETL marker genes is very disorganized in endosperms with a 2m : 2p genomic balance when compared with endosperms with a 4m : 1p genomic balance (figure 3b,c). While there are features of these endosperms that almost certainly result from parental conflict operating through imprinting (Haig & Westoby 1991; Scott *et al.* 1998), they also highlight the pivotal role played by maternally transmitted factors in establishing functional domains during early endosperm development. How this maternal control is exerted at a molecular level remains unclear, but data currently available suggest that it is achieved through a complex series of interactions between maternally inherited factors and imprinted genes activated after fertilization.

It is hoped that molecular analysis of the striking maternal effect mutants found in maize (Gavazzi *et al.* 1997; Evans & Kermicle 2001) and *Arabidopsis* (reviewed in Chaudhury & Berger 2001; Berger 2003) will help in understanding this process.

6. CONCLUSIONS

Little is currently known of the molecular and genetic mechanisms responsible for the endosperm acting as a hybridization barrier in plants. Gene dosage and imprinting effects in the endosperm are currently considered the 'gatekeepers' of endosperm development, yet conclusive evidence linking these processes with hybrid failure remains patchy. Analysis of the molecular mechanisms regulating endosperm development in hybrids will not only reveal the parts played by maternal determinants and parental imprinting in this complex process, but may also shed some light onto the evolutionary events that have led to the development, solely in the angiosperms, of this unusual triploid structure.

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GLOSSARY

- AMD: allelic message display
 BETL: basal endosperm transfer layer
 DAP: days after pollination
 FIS: fertilization-independent seed