Balance between maternal and paternal alleles sets the timing of resource accumulation in the maize endosperm

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Key aspects of seed development in flowering plants are held to be under epigenetic control and to have evolved as a result of conflict between the interests of the male and female gametes (kinship theory). Attempts to identify the genes involved have focused on imprinted sequences, although imprinting is only one mechanism by which male or female parental alleles may be exclusively expressed immediately post-fertilization. We have studied the expression of a subset of endosperm gene classes immediately following interploidy crosses in maize and show that departure from the normal 2 : 1 ratio between female and male genomes exerts a dramatic effect on the timing of expression of some, but not all, genes investigated. Paternal genomic excess prolongs the expression of early genes and delays accumulation of reserves, while maternal genomic excess foreshortens the expression period of early genes and dramatically brings forward endosperm maturation. Our data point to a striking interdependence between the phases of endosperm development, and are consonant with previous work from maize showing progression from cell proliferation to endoreduplication is regulated by the balance between maternal and paternal genomes, and from *Arabidopsis* suggesting that this ‘phasing’ is regulated by maternally expressed imprinted genes. Our findings are discussed in context of the kinship theory.

Keywords: endosperm; imprinting; interploidy crosses; kinship theory; maize (*Zea mays*); plant fertilization

1. INTRODUCTION

Maternal control of early seed development is held to be an important driver in the evolution of genes regulating flowering plant reproduction (Chaudhury & Berger 2001). As male and female gametic genomes can be considered to have differing evolutionary ‘interests’, a complex situation may pertain where conflict between these parental interests has resulted in the epigenetic modification of seed-expressed genes such that early development is regulated through a balance of maternally, paternally and biparentally expressed genes (Haig & Westoby 1989). This hypothesis requires that this epigenetic ‘marking’ (imprinting) should target genes regulating the provision of resources—but to date few have been identified. The *MEG1* gene of maize is an exception (Gutiérrez-Marcos *et al.* 2004), for it encodes a putative signal protein located at the basal transfer layer of the endosperm, which is required for grain filling as well as for correct development of the cell layer itself. This absence of imprinted genes involved in resource acquisition may be explained either by imprinting controlling major upstream regulators rather than physiologically functional genes or by the presence of other epigenetic systems that also modulate seed gene expression. Importantly, evolutionary fitness depends on the sum of these systems, so to identify the effects of the totality of these processes on seed fitness, we have

analysed the consequences of fertilization involving different numbers of complete genomes—so-called ‘unbalanced crosses’.

Flowering plants have double fertilization where fusion of sperm with the haploid egg and the diploid central cell generates the diploid zygote and triploid endosperm, respectively. The endosperm may either develop transiently, as in *Arabidopsis*, or be dispersed with the seed as in the grasses (Costa *et al.* 2004). A key feature of most endosperms is an initial period of nuclear division in the absence of cell walls. Several days after fertilization, these coenocytes cellularize and develop the characteristic ‘tissues’ of the endosperm (for maize: a central starchy region, a bounding layer of specialized aleurone cells, the basal endosperm transfer layer (BETL) through which resources are imported into the endosperm and an embryo-surrounding region (ESR) (Vijayaraghavan & Prabhakar 1984; Thompson *et al.* 2001; Costa *et al.* 2004)). Endosperm maturation is accompanied by significant levels of nuclear endoreduplication (Dilkes *et al.* 2002). Cooper (1951) showed the balance between the maternal and paternal genomic contributions to the endosperms of maize was critical to seed development, and any departure from a balanced cross between diploid parents resulted in kernel abortion. Subsequently, Lin (1984) was able to show that successful endosperm development in maize depended on the donation of two maternal and one paternal genomes, or multiples of this 2 : 1 ratio. Unlike in maize, interploidy crosses are fertile in *Arabidopsis*, but unbalanced transitory endosperms still differ strikingly from those resulting from balanced crosses, depending on the direction of the cross. Indeed,

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Dilkes *et al.* (2002) have shown that crosses between diploid and tetraploid lines within particular genotypes can result in lethality, and have identified the cause of this lethality to involve a pathway modified by the maternal effect transcription factor *TRANSPARENT TESTA GLABRA2* (*TG2*). However, in those interploidy crosses involving diploid, tetraploid and hexaploid lines giving rise to viable seeds, Scott *et al.* (1998) showed that donation of excess maternal genomes (maternal genomic excess: MGE) resulted in smaller seeds, while extra paternal genomes (paternal genomic excess: PGE) generated larger seeds. Cytological analyses of these seeds showed PGE resulted in an increased number of endosperm divisions and late cellularization, while MGE induced an abbreviated period of endosperm proliferation. These early data confirmed that maternal and paternal gametic genomes are not functionally equivalent, and must be differentially marked, or imprinted, during male and female gametogenesis.

Subsequently, many genes expressed during endosperm (but not embryo) development have been demonstrated to be imprinted, with only either the male or the female allele expressed (Köhler & Makarevich 2006; Huh *et al.* 2007). Importantly, many imprinted genes in both *Arabidopsis* and maize encode polycomb-group proteins, which operate in chromatin-remodelling complexes that regulate development through repression of target genes (Francis & Kingston 2001). For example, members of the *FERTILIZATION-INDEPENDENT SEED* (*FIS*) class of genes, which include *MEDEA* (*MEA*), *FIS2* and *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*), are imprinted in *Arabidopsis*, and *FIS* proteins form part of the plant polycomb-repressive complex (PRC) that regulates endosperm development (Grossniklaus *et al.* 1998; Luo *et al.* 1999; Ohad *et al.* 1999; Chanvivattana *et al.* 2004). Endosperm development in *fis* mutants commences normally, but the transition from the coenocytic to cellular stage, when functional domains of the endosperm are established, is disrupted, suggesting that the PRC regulates the ontogenetic sequencing of endosperm development, but not the events themselves (Ingouff *et al.* 2005).

Specific genes, imprinted or otherwise, have yet to be identified as responsible for aberrant development following interploidy crosses in maize, but a study of cell cycle progression (Leblanc *et al.* 2002) has shown MGE to shorten the early proliferation phase by the precocious induction of programmed cell death, and PGE to directly disrupt endoreduplication which characterizes maturation of the endosperm. Leblanc *et al.* (2002) suggest that *CYCZme1* (a mitotic B1 cyclin), *ZmWEE1* (a CDK kinase) and RB-like proteins may play key roles in the establishment of endoreduplication following proliferation, and thus are likely to be affected in interploidy crosses. Combining data on early development both from plants and animals, von Wangenheim & Peterson (2004) propose that endosperm development is regulated by an intracellular timer of differentiation rather than an imprinting-based system. This 'timer' is held to pace cell division on the basis of cytoplasmic growth, and is capable of counting meiotic cycles.

While PGE and MGE clearly affect the progression of endosperm development in species both with transitory (*Arabidopsis*) and persistent (maize) endosperms, it is

unclear how these differences are manifest at the transcriptional level—and particularly in terms of the expression of sequences associated with the accumulation of reserves that the kinship theory predicts should be the targets of parental conflict. We have therefore measured changes in the timing and levels of transcription of a selected subset of genes following reciprocal interploidy crosses. At very early stages in endosperm development, we show that MGE brings forward the expression of genes responsible for reserve accumulation, and shortens the expression period of early-expressed factors held to be responsible for endosperm patterning, while PGE delays expression of genes regulating reserve acquisition, and prolongs expression of early-expressed sequences. Importantly, both MGE and PGE fail to affect the timing of expression of most endosperm-expressed genes, and although imprinting must certainly play a role in the changes observed, very few of the genes affected are themselves imprinted.

2. MATERIAL AND METHODS

(a) *Plant material*

Diploid and tetraploid W23 inbred lines of maize (*Zea mays*) were greenhouse grown under a 16 h daylight cycle with supplementary lighting, at 22–28°C (day) and 16–20°C (night). Plants were watered to capacity and pollinations were carried out as described in *The maize handbook* (Freeling & Walbot 1996).

To confirm the ploidy of individual diploid and tetraploid lines, leaf material was analysed using flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA) as described by Pennington (2005). Peaks were measured using CELLQUEST software (BD Biosciences).

(b) *Endosperm isolation*

Endosperms were excised from kernels at 5 days after pollination (DAP) by microdissection according to Pennington (2005). Endosperm forms by far the major component of the kernel at 10 and 15 DAP; for this reason, entire kernels were sampled at these later time points.

(c) *Semiquantitative reverse transcription–polymerase chain reaction analysis*

RNA was extracted (TRIzol Reagent, Invitrogen, 15596-018) with additional DNase treatment (TURBO DNA-free, Ambion, AM1907) from each sample. For cDNA synthesis, 1 µg of each purified total RNA was used (RETROscript Kit, Ambion, AM1710). PCR reactions were carried out in 50 µl volume reactions, with 1 µl of 10 times diluted cDNA templates. The PCR amplifications were performed with the following programme: one step of 1 min at 95°C, 25, 30 and/or 35 cycles of 30 s at 95°C, 30 s at 58°C and 30 s or 1 min at 72°C (depends on different genes and fragment lengths) and a final step of 5 min at 72°C. PCR-amplified products were electrophoresed in 1.5 per cent agarose gels for approximately 40 min at 160 V and images taken using a UV transilluminator. All the reactions were repeated twice. Primers used for the amplification are listed in electronic supplementary material, table S1.

(d) *Real-time quantitative polymerase chain reaction*

Real-time quantitative PCR (qPCR) was carried out in triplicate with SYBR Green PCR Master Mix (ABsolute; Blue QPCR SYBR Green ROX Mix, Thermo SCIENTIFIC,

ABgene Nucleic Acid Amplification, AB-4162/A) using an Applied Biosystems Prism-7300. Levels of specific mRNA and maize *Actin1* control were calculated by the standard curve method (Applied Biosystems User Bulletin 2). The relative expression (arbitrary units) of each gene of interest was obtained by dividing by the equivalent *Actin1* expression. No-template controls were included as negative controls for each set of reactions. Three independent biological repeats gave similar results. Gene-specific primers are listed in electronic supplementary material, table S2.

3. RESULTS

Development of endosperm tissue domains following balanced crosses has already been reported (Thompson *et al.* 2001; Pennington *et al.* 2008), and although maize kernels abort following unbalanced interploidy crosses (Cooper 1951; Lin 1984), they remain viable until 20–30 DAP during which time the key endosperm tissue domains develop, but to different extents and at different rates (Pennington *et al.* 2008). We thus selected time points for our gene expression study covering the period during which the endosperm tissue domains are ‘mapped out’, but prior to any indication of tissue necrosis. Tetraploid and diploid lines W23 of maize were reciprocally pollinated and RNA extracted at 5, 10 and 15 DAP, as described in Pennington *et al.* (2008). As controls, similar extracts were made from endosperms resulting from diploid and tetraploid balanced crosses.

To determine whether observed differences in the development of the endosperm tissue domains were reflected in gene expression, genes were selected from the literature and available databases representing genes expressed early, late and throughout kernel development, genes expressed in key tissue domains and genes known to be imprinted (table 1). Only genes identified in maize and for which expression data have been reported were selected, for, while a more comprehensive list of sequences could have been made using sequences with homology to Arabidopsis endosperm-expressed genes, the striking level of neo- and subfunctionalization that has occurred during evolution of the gramineae (Springer *et al.* 2002; Danilevskaya *et al.* 2003; Gutiérrez-Marcos *et al.* 2006) suggests that this would introduce an unacceptable level of error into our data. Semiquantitative RT-PCR (25, 30 and/or 35 cycles) was carried out for each gene using extracted RNA samples from at least two biological replicates for each time point. Representative RT-PCR results are shown in figure 1, and others are shown in electronic supplementary material, figure S1, while the overall effect of parental imbalance on expression patterns is set out in figure 2.

The expression level and timing of a significant proportion of the genes studied were unaffected by paternal or maternal excess; this group of genes included ‘marker’ sequences for the aleurone (*VP1*, *CR4* and *DEK*) and *SAL1*, a Class E vacuolar-sorting protein. None of these genes is expressed solely in the endosperm (McCarty *et al.* 1991; Becraft *et al.* 1996; Lid *et al.* 2002; Shen *et al.* 2003). Ubiquitously expressed *FIE2* and *MEZ1* and endosperm-specific *FIE1* are reported to be imprinted in maize, expressed only from the female alleles (Haun *et al.* 2007; Hermon *et al.* 2007), and it is striking

that expression of *FIE2* and *MEZ1* is unaffected under conditions of MGE and PGE (as is almost certainly *FIE1*, although there is some intersample variation between 5 DAP $4n \times 2n$ endosperms following 25 and 30 PCR cycles). This similarity of expression levels is also unexpected in view of the presence of two extra sets of female alleles in MGE.

Two ‘early’ genes, *EBE-1* and *EBE-2*, similar to the DUF239 proteins of Arabidopsis (Magnard *et al.* 2003), are confirmed by our data to be expressed early in development, being represented in the 15 and 10 DAP samples. *EBE-2*, and to a lesser extent *EBE-1*, expression is affected by parental imbalance; following $4n \times 2n$ crosses, expression is suppressed in the 10 DAP sample, whereas after $2n \times 4n$ pollinations, *EBE-2* expression persists until 15 DAP (as also does expression of *EBE-1*, but at the limits of detection by semiquantitative RT-PCR). ‘Late’ genes, *OPAQUE2* and γ -ZEIN, principally involved in reserve accumulation in the central endosperm (Vicente-Carbajosa *et al.* 1997; Woo *et al.* 2001), are expressed between 10 and 15 DAP in balanced crosses, but are delayed in their expression to 15 DAP in PGE, and expressed as early as 5 DAP in MGE. *MRP1*, an imprinted ‘late gene’ encoding a putative transcription factor (Guo *et al.* 2003), behaves similarly. Two genes, the BETL-specific *MEG1* (Gutiérrez-Marcos *et al.* 2004) and *MRP1*, a transcription factor believed to regulate it (Gómez *et al.* 2002), exhibited similar but phased expression patterns. *MRP1* expression peaked at 5 DAP, and *MEG1* at 10 DAP in balanced crosses, but following $2n \times 4n$ pollinations, *MRP1* expression continued unchanged while *MEG1* expression increased from 5 to 10 DAP, and thereafter remained constant. $4n \times 2n$ pollinations resulted in high expression of *MRP1* and *MEG1* at 5 DAP, followed by a dramatic fall to undetectable levels by 10 DAP. However, by 15 DAP, transcripts of both genes were again detectable.

Although the RT-PCRs were repeated using biological replicates, we considered the errors inherent in the method (exemplified by the 25 and 30 cycle samples of $4n \times 2n$ 5 DAP samples of *FIE1*) required that our data be further validated. Real-time qPCR was therefore used to investigate the expression of genes representative of the three general classes of expression pattern; those unaffected by parental imbalance (*MEZ1*), early genes for which the expression period is ‘shifted’ by parental excess (*EBE-2*) and later-expressed genes that are similarly affected (*OPAQUE2*). The results shown in electronic supplementary material, figure S2, confirm that expression patterns revealed by qPCR reflect very closely those of the semiquantitative RT-PCR data, confirming the validity of this latter dataset. ANOVA analysis of the qPCR data followed by Tukey’s pairwise testing (electronic supplementary material, figure S2) highlights the striking difference between expression of *EBE-2* in $2n \times 4n$ and $4n \times 2n$ endosperms at 10 and 15 DAP, and for *OPAQUE2*, the difference between $2n \times 4n$ and $4n \times 2n$ at 5 DAP and the low expression in $2n \times 4n$ endosperms at 10 DAP. Both the semiquantitative RT-PCR and the qPCR confirm that although the timing of transcription varies for many of the genes studied, the overall level of expression of each gene in MGE and PGE is very similar to that occurring in balanced crosses.

Table 1. Genes involved in maize endosperm development.

gene	function	timing of expression in endosperm	reference
<i>CR4</i>	encodes a receptor-like kinase that is implicated in aleurone cell fate specification	expressed throughout the plant	Becraft <i>et al.</i> (1996)
<i>DEK1</i>	maintain and restrict aleurone cell fate imposed by <i>CR4</i>	expressed throughout the plant	Lid <i>et al.</i> (2002)
<i>EBE-1</i>	potentially involved in development of endosperm domains	commences in the central cell, maternally expressed in endosperm; peaks at 7 DAP	Magnard <i>et al.</i> (2003)
<i>EBE-2</i>	as <i>EBE-1</i>	expression 10× less than <i>EBE-2</i> as <i>EBE-1</i> , but no parent-of-origin effect	Magnard <i>et al.</i> (2003)
<i>FIE1</i>	maize homologue of the Arabidopsis polycomb-group gene <i>FIE</i>	endosperm-specific, maternally expressed 2–10 DAP	Hermon <i>et al.</i> (2007)
<i>FIE2</i>	as <i>FIE1</i> likely to be the functional orthologue of Arabidopsis <i>FIE</i>	bi-allelic expression in the embryo, and later in the endosperm. <i>FIE2</i> mRNA levels are significantly lower than those of <i>FIE1</i>	Hermon <i>et al.</i> (2007)
<i>MEG1</i>	may function in regulating nutrient trafficking into endosperm	maternally expressed in the BETL of the endosperm. BETL marker	Gutiérrez-Marcos <i>et al.</i> (2004)
<i>MEZ1</i>	putative histone methyltransferase related to the <i>Drosophila PcG</i> gene	maternally expressed in endosperm tissue, but bi-allelic elsewhere	Haun <i>et al.</i> (2007)
<i>MRP1</i>	a transfer cell-specific MYB-related transcriptional activator	expressed at 3 DAP; maximum at 11 DAP; decreasing after 16 DAP	Gómez <i>et al.</i> (2002)
<i>NRP1</i>	putative transcription factor	maternally expressed only in the endosperm after fertilization	Guo <i>et al.</i> (2003)
<i>OPAQUE2</i>	activates <i>alpha-zein</i> class of storage proteins	expressed in the endosperm subaleurone layer from 10 DAP	Vicente-Carbajosa <i>et al.</i> (1997)
<i>PBF</i>	member of the Dof class of plant Cys2–Cys2 zinc-finger DNA-binding proteins	endosperm specific; 10–15 DAP	Vicente-Carbajosa <i>et al.</i> (1997)
<i>SAL1</i>	vacuolar protein sorting gene implicated in membrane vesicle trafficking	expressed throughout the plant	Shen <i>et al.</i> (2003)
<i>VP1</i>	transcriptional activator required for seed maturation	expressed in embryos at 10 DAP, peaking at 16 DAP and then decreasing gradually. Aleurone/embryo marker	McCarty <i>et al.</i> (1991)
γ -ZEIN	endosperm-specific storage protein	expressed throughout the endosperm by 10 and 15 DAP	Woo <i>et al.</i> (2001)

4. DISCUSSION

Both Arabidopsis and maize show dramatic parental excess phenotypes. The large seeds that result from pollination by a $4n$ or $6n$ male parent in Arabidopsis show an extended phase of endosperm proliferation such that after 5 DAP endosperms contain twice the number of endosperm nuclei as are formed after a $2n \times 2n$ balanced cross (Scott *et al.* 1998). Cellularization is also significantly delayed in these seeds. Pollination of polyploid females by diploid pollen results in small seeds, containing half the numbers of endosperm nuclei at 5 DAP, and in which cellularization is brought forward. Although potentially confounded by abortion of interploidy kernels late in development (20+ DAP), data from early maize endosperms show very different phenotypes depending on the direction of the interploidy cross (Cooper 1951; Charlton *et al.* 1995; Pennington *et al.* 2008). $4n \times 2n$ pollinations result in comparatively normal development of the endosperm and embryo over the first 15 DAP, with correct formation of the aleurone, BETL and ESR domains. However, the kernels are smaller than that of wild-type, contain fewer endosperm cells (Pennington

2005) and accumulate starch between 8 and 10 DAP, far earlier than wild-type (normally 10–14 DAP) (Pennington *et al.* 2008). Patterning of the endosperm is strongly affected in $2n \times 4n$ crosses with disruption of the aleurone and BETL domains. The endosperm itself, although larger and containing more cells (Pennington 2005), becomes misshapen by 18–20 DAP (Pennington *et al.* 2008).

A preliminary RT-PCR study of endosperm gene expression, 4, 8 and 10 DAP, revealed changes in the timing of gene expression, rather than in transcript levels (Pennington *et al.* 2008), suggesting that the dramatic phenotypic differences between reciprocal interploidy crosses may be reflected in the phasing of gene expression programmes. Our data confirm this but, importantly, also reveal that only a subset of genes are involved; for example, genes such as *CR4*, *DEK1* and *VP1*, which are crucial for endosperm formation and are activated at different phases of its development, remain unaffected by an excess of either maternal or paternal alleles. Also unaffected are the majority of imprinted genes encoding polycomb-group proteins,

Figure 1. The effect of male and female genomic excess on endosperm gene expression. Semiquantitative RT-PCR on 5, 10 and 15 DAP maize endosperm. (a–e) *CR4*, *EBE-2*, *FIE1*, *MRP1* and *OPAQUE2* genes, respectively. (f) *Actin1*: internal control. For *CR4*, *FIE1* and *MRP1* genes, 25 and 30 cycles were used in each time point, 30–35 cycles were used for *EBE-2* and *OPAQUE2*. Two replicates were carried out for all genes. The full semiquantitative RT-PCR dataset is shown in electronic supplementary material, figure S1.

some of which are expressed only in the early stages of endosperm development. The fact that both bi-allelically expressed and imprinted sequences are expressed at equivalent levels (as determined by our methods) in PGE, MGE and balanced crosses confirms the presence of a highly effective dosage compensation system capable of regulating transcript levels.

We show that two early genes (*EBE-1*, *EBE-2*) are affected by genomic excess, maternal genomes curtailing and paternal genomes prolonging the period of their expression. Both genes are reported to function in early endosperm domain specialization, with *ZmEBE-1* expressed from the maternal allele in the young endosperm (Magnard *et al.* 2003). By contrast, later-expressed genes involved in accumulation of resources are affected by genomic excess in a reciprocal fashion. Thus, expression of *OPAQUE2*, which regulates the expression of zein storage proteins (Vicente-Carbajosa *et al.* 1997), and the γ -ZEIN sequences themselves (Woo *et al.* 2001) are restricted to a period late in development in the presence of excess paternal genomes, while maternal excess extends the period of expression, bringing it forward significantly. The data generally confirm the structural observations from both Arabidopsis and maize and support the kinship theory (Haig & Westoby 1991; Haig 2000) which holds that the interest of the maternal genome will be in restricting endosperm development, evidenced by the curtailing of expression of early genes with roles in establishing the tissue plan of the endosperm, and the early and extended expression of the maturity phase, characterized by the

accumulation of reserves. The kinship theory also predicts that the interest of the paternal genome would be opposite, promoting the establishment phase—creating the possibility of developing a larger embryo, and postponing the maturity phase (after which expansion growth is no longer possible).

The expression patterns of three genes require further consideration. The imprinted transcription factor *NRPI*, which is normally expressed 15–25 DAP (Guo *et al.* 2003), responds similarly to *OPAQUE2* and γ -ZEIN, suggesting that it is a component of the gene network regulating maturation of the kernel. The expression of *MRP1*, a transcription factor regulating genes in the BETL (Gómez *et al.* 2002), and *MEG1*, a putative signalling protein synthesized in the BETL and regulated by *MRP1* (Gutiérrez-Marcos *et al.* 2004), normally peaks at about 5 and 10 DAP, respectively—consistent with *MRP1* activating *MEG1*. Paternal excess results in continued accumulation of transcript throughout development, while maternal excess causes expression of both genes to cease at 10 DAP, only to recommence at a lower level by 15 DAP. Both genes are involved in patterning the developing endosperm, and thus would be expected to be promoted by the paternal and suppressed by the maternal genome. However, the reappearance of both transcripts after 15 DAP in MGE suggests that they may play roles in both BETL establishment and subsequently its function as a transfer layer during the accumulation of resources that characterizes endosperm maturation. We find no evidence that imprinting *per se* determines whether expression of a gene is affected by genomic imbalance.

Figure 2. Diagrammatic summary of gene expression changes in maize endosperms following interploidy crosses. (a) Marker genes; *CR4*, *DEK1*, *SAL1* & *VP1* show no difference in expression; (b) Imprinted Polycomb Group genes; *FIE1*, *FIE2* & *MEZ1* show little or no difference in expression; (c) Early expressed genes; *EBE-1* & *EBE-2* show premature expression termination in MGE compared with PGE where expression is prolonged. Two interacting genes; *MRP1* & *MEG1* expression decreases at 10 DAP only to be restored at 15 DAP in MGE, compared with a progressive increase in PGE; (d) Genes involved in resource accumulation; *OPAQUE2* & γ -ZEIN expression is brought forward to 5 DAP in MGE compared with a delay in expression in PGE. Transcription factors; *NRPI* & *PBF*, show a similar expression pattern to *OPAQUE2* and γ -ZEIN. Green lines (balanced cross), $2n \times 2n$ ($4n \times 4n$ data not shown); pink lines (MGE), $4n \times 2n$; blue lines (PGE), $2n \times 4n$.

While the changes in expression timing of our early and late genes map closely onto the changes in progression from the ‘proliferation’ to the ‘endoreduplicative’ phases of endosperm development described by Leblanc *et al.* (2002), it is not clear how alteration to the expression of cell cycle progression genes such as *CYCZme1*, *ZmWEE1* or RB-like sequences could modulate expression of genes such as *OPAQUE2*, and leave the majority of other genes unaffected. Perhaps, more likely is that parental genomic balance affects the activity of major regulatory genes, which control integrated gene networks involving many facets of development. *FIS* genes are promising candidates for these ‘regulatory genes’, but *fis* mutant phenotypes are unavailable for maize, and at least one member of the PRC chromatin-remodelling complex, FIE, has become duplicated and sub- or neo-functionalized in maize (Danilevskaya *et al.* 2003; Gutiérrez-Marcos *et al.* 2006). Nevertheless, mutations at *fie* loci in Arabidopsis result in heterochronic disruption of endosperm development, particularly the progression from coenocytic to cellular development (Ingouff *et al.* 2005), and it is interesting that in PGE maize endosperms, where the representation of active, maternally expressed *FIS* loci would be attenuated, the

expression of early genes (*EBE-1*, *EBE-2*) is significantly prolonged. Zein accumulation in these endosperms is also delayed, pointing to an interdependence between early development (which in Arabidopsis primarily involves cell proliferation) and the later accumulation of reserves. MGE appears to reverse this pattern, indicating that an excess of active *FIS* alleles results in the PRC inducing maturation events (including reserve accumulation) very early, at the expense of the proliferation phase. Thus, as proposed by Ingouff *et al.* (2005) for Arabidopsis, in maize it may also be that the PRC regulates the transition from proliferation to maturation, and increasing dosages of maternally expressed *FIS* sequences result in the increasing dominance of the later maturation phase.

However, it is unlikely that the PRC is the sole regulator of endosperm development, repressing the early proliferative phases and promoting maturation and reserve accumulation, for the kinship theory suggests that paternal alleles should promote the proliferation phase of development, which sets the final size of the endosperm. Further, the mosaic of epigenetic information carried by the male and female gametic genomes is likely to be complex and comprise not only imprinting marks, but systems of dosage compensation,

and marking patterns that differentiate male from female chromosomes that enable a measured activation of the paternal genome post-fertilization (Vielle-Calzada *et al.* 2000; Scholten *et al.* 2002; Mosher *et al.* 2009). An understanding of the epigenetic regulation of endosperm development, and indeed the validity of the kinship theory, must thus await unequivocal data on the overall maternal, paternal and bi-allelic input into early seed development and its consequences in terms of the expression of genes that directly results in the complex phenotypes involved.

The kinship theory holds that the evolutionary interests of the male genome will be for the formation of a larger and very fit endosperm, and for the female genome a successful endosperm—but of fitness equivalent to its immediate sibs. Within its limits, our data support this assertion, and reveal that this is achieved not principally by altering the expression levels of large numbers of genes, but rather by modulating the timing of expression of a relatively small number of key sequences.

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