Parent-of-origin effects on seed development in *Arabidopsis thaliana*

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SUMMARY

Many flowering plants are polyploid, but crosses between individuals of different ploidies produce seeds that develop abnormally and usually abort. Often, seeds from interploidy crosses develop differently depending on whether the mother or father contributes more chromosome sets, suggesting that maternal and paternal genomes are not functionally equivalent. Here we present the first cytological investigation of seed development following interploidy crosses in *Arabidopsis thaliana*. We find that crosses between diploid and tetraploid plants in either direction, resulting in double the normal dose of maternal or paternal genomes in the seed, produce viable seeds containing triploid embryos. However, development of the seed and in particular the endosperm is abnormal, with maternal and paternal genomic excess producing complementary phenotypes. A double dose of maternal genomes with respect to paternal contribution inhibits endosperm development and ultimately produces a smaller embryo. In contrast, a double dose of paternal genomes promotes growth of the endosperm and embryo. Reciprocal crosses between diploids and hexaploids, resulting in a triple dose of maternal or paternal genomes, produce seeds that begin development with similar but more extreme phenotypes than those with a double dose, but these invariably abort. One explanation of our observations is that seeds with maternal or paternal excess contain different doses of maternally or paternally expressed imprinted loci affecting endosperm development.

Key words: seed development, endosperm, interploidy crosses, cell cycle, parental imprinting, *Arabidopsis, tetraspore*

INTRODUCTION

Although flowering plants easily tolerate polyploidy (Leitch and Bennett, 1997), crosses between individuals of different ploidies often result in abnormal seed development followed by abortion. This phenomenon has been investigated through interploidy crosses between closely related species, or within species using diploids and their autopolyploids. The seeds of flowering plants contain two fertilisation products, embryo and endosperm, and interploidy crosses disturb the genetic constitution of both of these. The embryo results from fusion of a sperm and an egg, while the primary endosperm nucleus is produced by fusion of a sperm and two polar nuclei (which like the egg are derivatives of the megaspore). In most species, the endosperm carries the same alleles as the embryo, but contains an extra maternally derived genome (Friedman, 1995).

Developing seeds from reciprocal interploidy crosses often show different phenotypes depending on which parent contributed more chromosomes. The visible differences following interploidy crosses are often much more dramatic in the endosperm, which transmits nutrients from the seed parent to the developing embryo (Brink and Cooper, 1947; Maheshwari, 1950; Bhatnagar and Sawhney, 1981; Vijayaraghavan and Prabhakar, 1984; Lopes and Larkins, 1993), than in the embryo itself. The role of the endosperm in seed development, combined with the phenotypes observed, strongly suggests that endosperm failure is the primary cause of seed abortion following interploidy crosses (Thompson, 1930; Watkins, 1932; Müntzig, 1933; Cooper and Brink, 1945; Brink and Cooper, 1947; Woodell and Valentine, 1961; Johnston et al., 1980; Kermicle and Alleman, 1990; Haig and Westoby, 1991; Birchler, 1993).

A variety of hypotheses have been offered to explain the data from interploidy crossing studies (reviewed by Haig and Westoby, 1991; Birchler, 1993). Historically, many of these focused on the genomic constitutions of the embryo, endosperm, and/or sporophytic tissues of the seed parent, which in a normal 2x × 2x cross (i.e. between two diploid plants) are 2x, 3x, and 2x respectively. Some researchers proposed that abnormal ploidy ratios between embryo and endosperm, endosperm and sporophytic tissues, or among all three are responsible for disturbances to seed development (Watkins, 1932; Müntzig, 1933; Cooper and Brink, 1945). Others concentrated on the endosperm, arguing that a 3x ploidy (Sarkar and Coe, 1971) or a ratio of 2 maternal genomes (2m) to 1 paternal genome (1p) (Nishiyama and Inomata, 1966) is critical for normal seed development. In some genera,
interspecific interploidy crosses have greater success than same-ploidy crosses; Johnston et al. (1980) accounted for this with the Endosperm Balance Number (EBN) hypothesis, according to which a 2m:1p ratio of effective (not necessarily absolute) ploidy levels in the endosperm is needed. The conflicting hypotheses were definitively tested by Lin (1984) through a series of crosses in maize, using 2x or 4x pollen parents, and 2x seed parents that contributed varying numbers of polar nuclei to the endosperm due to the *indeterminate gametophyte* (*ig*) mutation. Thus Lin generated seeds with 2x sporophytic tissues and either 2x or 3x embryos, but with a range of endosperm karyotypes that varied both in total ploidy and in the balance of maternal to paternal genomes. Lin concluded that a 2m:1p genomic ratio rather than a specific ploidy is the critical factor for normal endosperm, and also that the genomic ratios among endosperm, embryo, and/or sporophytic tissues are irrelevant to seed development.

Lin (1984) interpreted the 2m:1p requirement as an indication that parentally imprinted genes are involved in development of the endosperm. Imprinting refers to the epigenetic modification of loci resulting in differential expression depending on parent of origin. This phenomenon is best understood in mammals, where experiments in mouse to alter parent-specific dosage of chromosome arms and whole genomes have established that a 1m:1p ratio is required for development of a viable embryo, most likely due to the cumulative actions of imprinted loci in several regions of the mouse genome (Surani et al., 1990; Cattanach and Beechey, 1997). So far more than 15 imprinted genes have been cloned from mouse and human, and their uniparental expression confirmed by molecular analysis (John and Surani, 1996). In mammals, imprinted genes are differentially expressed in the embryo and its derivatives, but genetic and molecular evidence indicates that in flowering plants imprinted genes function primarily (if not exclusively) in the endosperm. While mammalian embryos require both maternal and paternal genomes, plant embryos can complete development with a constitution of 1m:0p or 2m:0p. In contrast, all sexually reproducing angiosperms need maternal and paternal contributions to the endosperm, and even seeds containing parthenogenetic embryos sometimes require fertilisation of the polar nuclei (Sarkar and Coe, 1966; Nogler, 1984; Kermicle and Alleman, 1990). Several chromosomal regions or individual loci have been identified in maize which confer different phenotypes on the endosperm depending on parent of origin rather than dosage; these include the *R* locus (Kermicle, 1970) and the long arm of chromosome 10 (Lin, 1982). Molecular evidence for imprinting in maize endosperm was provided by Lund et al. (1995), who found that some alleles of zein genes, which encode storage proteins, are only transcribed when maternally derived.

Imprinting has been particularly well studied in maize for several reasons, including its elegant genetics and the persistence of endosperm in the mature seed. However, parent-of-origin effects on endosperm development following interploidy crosses are also seen in plants where the endosperm is absorbed by the developing embryo, such as *Brassica* species (Håkansson, 1956; Nishiyama and Inomata, 1966). Therefore *Arabidopsis thaliana*, which is closely related to brassicas, might also be expected to show altered seed phenotypes when the parental genome ratio is disturbed. Our investigation of interploidy crossing behaviour in *Arabidopsis* grew out of research on the *tetraspore* (*tes*) mutant, which due to a defect in pollen development produces sperm with a range of ploidies (Spielman et al., 1997). Many of the seeds produced by *tes* mutants show features previously reported following 2x × 4x crosses in brassicas, suggesting that the abnormal phenotypes of *tes* seeds could result from inheritance of extra paternal genomes.

Here we present the first developmental study of seeds from interploidy crosses in *Arabidopsis*. In contrast to most species investigated, we find that *Arabidopsis* readily produces viable seeds containing 3x embryos from either 2x × 4x or 4x × 2x crosses. However, these seeds develop abnormally, with reciprocal crosses showing complementary phenotypes. Seeds from 2x × 6x and 6x × 2x crosses invariably abort, with even more extreme phenotypes. Seeds from self-pollinated *tes* mutants show a mixture of phenotypes resembling those resulting from 2x × 2x, 2x × 4x, and 2x × 6x crosses. From the evidence described below we can conclude that altering parental genome dosage in an *Arabidopsis* seed affects several aspects of the cell cycle in the endosperm, as well as endosperm differentiation. We also find that the size of a mature seed (and by inference the ability of endosperm to acquire resources from the seed parent) is dramatically affected by the relative maternal and paternal contribution. The complementary patterns of seed development following reciprocal interploidy crosses are consistent with the hypothesis that imprinted loci affect seed growth. Our results also support the parental conflict theory of imprinting (Haig and Westoby, 1989, 1991; Moore and Haig, 1991), according to which imprinting evolved due to competition between parental genomes over resource allocation to offspring.

**MATERIALS AND METHODS**

**Plant material**

Plants were grown at 22°C with a day length of 18 hours in a Fisons growth cabinet. The *Arabidopsis* ecotypes used were C24 (2x, 4x), Ler (2x, 4x), and Col-1 (6x). 4x Ler seeds were kindly donated by Daniel Perazza (Université Joseph Fourier, Grenoble, France) and 4x Arabidopsis ecotypes used were C24 (2x, 4x), and Col-1 (6x). 4x Ler seeds were kindly donated by Daniel Perazza (Université Joseph Fourier, Grenoble, France) and 4x Arabidopsis Stock Centre (USA); this seed yielded a mixture of 4x and 6x plants, so 6x plants were identified by chromosome squashes before crosses were performed.

The C24 (2x) plants were hemizygous for the A9-barnase transgene conferring male sterility (Paul et al., 1992); these segregate 1:1 male-sterile and male-fertile plants. The male-sterile segregants were used as seed parents in crosses since emasculation is not required. The male-fertile segregants were used as pollen parents.

**Cross pollinations**

Crossovers between individual *Arabidopsis* plants were made in one of two ways. Where the seed parent was male fertile, flowers were emasculated 1 day prior to anthesis and pollinated the following day. The remainder of the inflorescence was removed to prevent self-fertilisation. Where the seed parent was male sterile (A9-barnase), open flowers were pollinated without emasculation. Developing seeds were collected for microscopy at 2 to 7 days after pollination and processed as described below. Mature seeds were collected when pods were desiccated. Seed weight was measured using a Cahn C-31 microbalance (Cahn Instruments Inc., Cerritos, CA, USA).
Confocal laser scanning microscopy
Specimens were prepared according to Braselton et al. (1996) and imaged at the University of Leicester (UK) using an MRC 600 confocal laser scanning microscope controlled by COMOS software (Bio-Rad Microscience, Hemel Hempstead, UK). Feulgen-stained specimens were excited using an argon ion laser at 488 nm, and emissions detected at ≥515 nm. Images were collected using a Kalman filter, with an image size of 768×512 pixels.

Chromosome counts
Chromosomes were counted using the method of Bailey and Stace (1992). The ploidy of seed and pollen parents was established by examining immature inflorescences prior to making crosses. The ploidy of embryos in F1 seeds was determined by germinating seeds and preparing chromosome squashes from shoot apices of 5- to 7-day-old seedlings.

Seed clearing
To clear seeds for light microscopy, seeds were dissected from siliques in a drop of 1 M KOH on a microscope slide under a dissecting microscope. The pod walls were removed and a coverslip applied. Embryos were released from the seed coat by applying gentle pressure to the coverslip. Whole-mount preparations were examined using a Zeiss Axioskop microscope using dark-field illumination and phase contrast.

Image processing
35 mm transparencies or negatives were scanned using a Polaroid Printscanner. Confocal images were converted to TIFF files. Images were processed for publication using Adobe Photoshop 3.0.

RESULTS

Arabidopsis seeds can complete development with a double but not triple dose of maternal or paternal genomes
To test the effect on seed development of varying parental genome dosage, we performed two series of cross pollinations, using diploid and autopolyploid Arabidopsis. For balanced crosses, 2x×2x, 4x×4x, and 6x×6x crosses were performed manually. In these crosses, embryo and endosperm ploidy could be increased without disrupting the normal maternal: paternal genome balance. For interploidy crosses, which alter both ploidy and parental genome balance, two sets of reciprocal cross pollinations were performed, using 2x and 4x or 2x and 6x parents. The predicted genomic compositions of embryo and endosperm for all crosses performed are shown in Table 1. Seed viability was assayed by germination on soil, and ploidy of germinated seedlings was checked using chromosome squashes (Table 1; Fig. 1).

Seed set following interploidy crosses in Arabidopsis, though not seed development, was previously investigated by Rédei (1964). As in the earlier report, our crosses between 2x and 6x plants failed to produce viable seed in either direction, while 4x×2x crosses yielded a high proportion of viable seed (nearly all seeds were plump, and 19 germinated out of 20 planted). However, although Rédei found that only 2.7% of seeds from 2x×4x crosses appeared normal, in our 2x×4x cross nearly all seeds were well filled, and 18 of 20 germinated. All seedlings grown from our 4x×2x and 2x×4x crosses were 3x, confirming that they were the progeny of 2x and 4x parents.

Table 1. Outcome of balanced and interploidy crosses

<table>
<thead>
<tr>
<th>Seed parent</th>
<th>Pollen parent</th>
<th>Predicted embryo ploidy (m:p)</th>
<th>Predicted endosperm ploidy (m:p)</th>
<th>Mean no. seeds/silique ± s.d.</th>
<th>% seed germinated (no. seeds)</th>
<th>Seedling ploidy (no. seedlings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced embryo and endosperm</td>
<td>2x</td>
<td>2x</td>
<td>2x</td>
<td>3x</td>
<td>49.8±10.9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4x</td>
<td>4x</td>
<td>4x</td>
<td>6x</td>
<td>39.7±9.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6x</td>
<td>6x</td>
<td>6x</td>
<td>9x</td>
<td>36.3±8.0</td>
<td>95</td>
</tr>
<tr>
<td>Maternal excess</td>
<td>4x</td>
<td>2x</td>
<td>3x</td>
<td>5x</td>
<td>45.3±9.1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>6x</td>
<td>2x</td>
<td>4x</td>
<td>7x</td>
<td>37.0±9.4</td>
<td>0</td>
</tr>
<tr>
<td>Paternal excess</td>
<td>2x</td>
<td>4x</td>
<td>3x</td>
<td>4x</td>
<td>47.3±9.9</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>2x</td>
<td>6x</td>
<td>4x</td>
<td>5x</td>
<td>47.8±11.3</td>
<td>0</td>
</tr>
</tbody>
</table>
Light micrographs of developing embryos (Fig. 1) show that in all crosses, embryos follow the normal stages of embryogenesis, but in lethal interploidy crosses (6x · 2x and 2x · 6x), embryos arrest at globular to heart stage. In the viable interploidy crosses (4x · 2x and 2x · 4x) embryo size is affected, with 3x embryos from 2x × 4x crosses becoming larger even than 4x embryos from balanced 4x × 4x crosses, and 3x embryos from 4x × 2x crosses remaining smaller even than 2x embryos from balanced 2x × 2x crosses. The size of mature seeds is affected both by absolute number of genomes and parental genome balance. Balanced crosses all produce 1m:1p embryos and 2m:1p endosperms, but seed size and dry weight increase with total ploidy, as previously reported (Koornneef, 1994). However, our results show that maternal or paternal excess has a dramatic effect on seed growth, separate from the effect of varying the total number of genomes. Seeds from 4x × 2x crosses are lighter than seeds produced by diploids, despite containing more genomes, while 2x × 4x seeds are heavier than those produced by tetraploids and even hexaploids, despite containing fewer genomes (Fig. 2). On average, seeds from 4x × 2x crosses are only 27% of the weight of seeds from 2x × 4x crosses, despite containing an additional genome. Parental ecotype has a relatively small effect on seed weight compared to parental genome balance (Fig. 2). Seeds from the 6x × 2x and 2x × 6x crosses are not directly comparable with the others, as these die before completing development, and mature siliques contain only small, shrivelled seeds.

Self-pollinated tes plants produce a mixture of normal, large, and shrivelled seeds (Fig. 3), resembling those produced by 2x × 2x, 2x × 4x, and 2x × 6x crosses, respectively. This supports the hypothesis that some tes mutant seeds develop abnormally due to transmission of extra genomes by tes pollen (Spielman et al., 1997).

Genomic imbalance affects the cell cycle and differentiation in the developing endosperm

To further investigate the effects of altering ploidy and parental genome ratios in Arabidopsis, we used Feulgen staining combined with confocal microscopy to examine embryos and endosperms in intact fixed seeds. A representative seed from a 2x × 2x cross is shown in Fig. 4. Our observations of seed development in 2x × 2x crosses were similar to previous reports (Mansfield and Briarty, 1990a,b; Schneitz et al., 1995). After fertilisation, the primary endosperm nucleus undergoes several rounds of mitosis without cytokinesis before the zygote divides. Endosperm `protoplasts' (nuclei with associated cytoplasm) migrate to different regions of the embryo sac and proliferate, remaining coenocytic during early seed development. Mansfield and Briarty (1990a,b) identified two types of endosperm in

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**Fig. 1.** Seed development following balanced and interploidy crosses. Column 1: mature seeds from balanced crosses producing normal ratios of maternal to paternal genomes (2x × 2x, 4x × 4x, and 6x × 6x) and interploidy crosses producing seeds with maternal excess (6x × 2x, 4x × 2x) or paternal excess (2x × 4x, 2x × 6x). Insets: representative chromosome squashes from plants grown from these seeds. Chromosome counts: 2x × 2x, 2n=10; 4x × 4x, 2n=20; 6x × 6x, 2n=30; 4x × 2x, 2n=15; 2x × 4x, 2n=15. Columns 2-4: embryo development following the crosses. Embryos arrest by heart stage in both the 6x × 2x and 2x × 6x crosses. Scale bars, column 1, 1 mm; columns 2-4, 200 μm.
Arabidopsis, which they termed 'micropylar' and 'chalazal'. We have subdivided their 'micropylar' endosperm into central peripheral (denoting the regularly spaced protoplasts lining the central region of the embryo sac) and micropylar peripheral (which forms a denser tissue surrounding the suspensor and base of the embryo). While central peripheral endosperm nuclei remain well separated throughout development, the nuclei of micropylar peripheral and chalazal endosperm are embedded in a common cytoplasm at either pole of the embryo sac.

Fig. 2. Seed size variation in balanced and interploidy crosses. The mean dry seed weight for each set of crosses is represented by a bar; the range depicted for each bar shows the mean values for each ecotype combination. For each cross, the first letter represents the ecotype of the seed parent, and the second letter, the pollen parent. c, C24; l, Ler; o, Col-1.

Balanced crosses

By 4 DAP (days after pollination) in 2x × 2x crosses, the embryo is at the globular stage. The suspensor is sunk in micropylar peripheral endosperm, central peripheral endosperm lines the embryo sac, and the chalazal endosperm is a prominent multinucleate tissue. By 5 DAP the embryo has reached heart stage and the peripheral endosperm is cellularising. Near the chalazal end of the embryo sac, some peripheral endosperm protoplasts appear to take on characteristics of the chalazal endosperm, forming enlarged, densely cytoplasmic, and sometimes multinucleate 'nodules'. Similar endosperm nodules near the chalazal pole have been reported in other species (Håkansson, 1956; Schulz and Jensen, 1974; Bhatnagar and Sawhney, 1981; Vijayaraghavan and Prabhakar, 1984); their function is unknown. At 6 DAP, the embryo is at the torpedo stage; the micropylar peripheral endosperm appears flattened, but the chalazal endosperm remains. By 7 DAP, the embryo almost fills the seed, and the chalazal endosperm has disappeared.

The sequence and timing of events in polyploid balanced crosses are similar to those in the diploid, but chalazal endosperm and nodules are larger.

Interploidy crosses

By 4 DAP in 4x × 2x crosses, endosperm mitosis and embryo differentiation are slower than in balanced crosses. Endosperm cellularisation begins slightly early, between 4 and 5 DAP. At this time there are far fewer central peripheral endosperm protoplasts than in controls, resulting in a hypoplastic endosperm with a small number of large cells. The micropylar peripheral endosperm appears less dense than in balanced crosses. Chalazal endosperm remains binucleate and often begins to collapse after 5 DAP, although the embryo is not yet filling the seed. No chalazal nodules are seen.

Seeds from 6x × 2x crosses have a similar but more extreme phenotype than those from 4x × 2x crosses. The peripheral endosperm cellularises early, at 4 DAP, and embryos abort by globular to heart stage. No distinct micropylar peripheral endosperm is seen, and the chalazal endosperm disappears by 5 DAP.

Seeds with paternal excess show complementary phenotypes to those with maternal excess. In 2x × 4x crosses, embryos develop at about the same rate as in balanced crosses. However, endosperm development is marked by hyperplasia. The central peripheral endosperm undergoes accelerated mitosis, and may become convoluted, apparently to accommodate the large number of protoplasts formed. The endosperm also cellularises late, at 6 DAP, so that when cytokinesis occurs there are many, small endosperm cells. The micropylar peripheral endosperm is unusually vigorous, and begins to engulf the embryo, although it recedes after 6 DAP. The chalazal endosperm is often enlarged and vacuolate, and chalazal nodules may also be very large. In some seeds it appears that the chalazal endosperm collapses early but large nodules remain.

For the first few days, embryogenesis in 2x × 6x crosses proceeds at about the same rate as in 2x × 4x crosses, but never passes the heart stage. Hyperplasia of the endosperm is even more dramatic. The central peripheral endosperm divides
rapidly without ever cellularising, and may become highly convoluted. Both micropylar peripheral and chalazal endosperm, as well as chalazal nodules, become hugely overgrown and vacuolate, eventually engulfing the embryo and filling the seed.

tetraspore seeds
Seeds from self-pollinated tes mutants show a range of phenotypes similar to those from interploidy crosses with varying degrees of paternal excess, as well as from balanced crosses. This is consistent with the hypothesis that at least some tes seeds develop abnormally or abort because they contain extra paternal genomes. Fig. 7 shows two seeds from the same tes mutant silique, which resemble wild-type seeds from 2x × 2x crosses (Fig. 7A,B) and 2x × 6x crosses (Fig. 7C-E), respectively.

Rates of endosperm mitosis in balanced and interploidy crosses
Since parental genome imbalance has such a striking effect on endosperm proliferation, we further investigated this phenomenon by counting nuclei of the central peripheral endosperm. Several stages of seed development in balanced and interploidy crosses were examined, using serial optical sections generated by confocal microscopy. The results are shown in Fig. 8. Balanced crosses all show roughly the same rate of endosperm mitosis until 4 DAP. The numbers of nuclei in 2x × 2x endosperms increase rapidly between 4 and 5 DAP, then level off, concomitant with cellularisation (Figs 5, 6). Numbers of nuclei in 4x × 4x and 6x × 6x endosperms follow a similar pattern, but fewer (and larger) nuclei are ultimately produced. However, endosperms in seeds from interploidy crosses show very different patterns of proliferation, with maternal and paternal excess resulting in dramatically slowed and accelerated mitosis, respectively. For example, at 6 DAP, 4x × 2x endosperms contain a mean of 84 nuclei (s.e.m ± 14.3, n = 5), only about 20% of the number in 2x × 2x seeds at this time (429±30.7, n = 3); while 2x × 4x endosperms contain a mean of 630 nuclei (±26.6, n = 4), nearly 150% of the number in 2x × 2x seeds. Paternal excess also affects the duration of the mitotic phase, as numbers of nuclei in 2x × 4x endosperms continue to rise sharply after 5 DAP. There is only a very slight increase in numbers of nuclei after 5 DAP in 2x × 6x endosperms, but this is presumably because these seeds are dying.

DISCUSSION
The parental genome ratio in Arabidopsis seeds affects seed development, viability and size
Following interploidy crosses in Arabidopsis, we find that seeds with double the normal dose of paternal genomes (relative to maternal genomes) show accelerated mitosis and delayed cellularisation of the endosperm, and are abnormally large at maturity. In contrast, those containing a double dose of maternal genomes exhibit reduced endosperm mitosis and precocious cellularisation, and are abnormally small. Seeds with a triple dose of either paternal or maternal genomes begin development with similar phenotypes to those with a double dose, but abort when the embryo is at globular to heart stage.

Interploidy crosses change the nuclear genetic composition of the embryo and endosperm in two dimensions, the overall ploidy (x) and the ratio of maternally to paternally derived genomes (m:p). There are two lines of evidence that the phenotypes we observe depend on the parental genome ratio in the seed, rather than the total genome dosage in the endosperm and/or the embryo. The first is provided by examining the effects of changing one dimension while holding the other constant. The second comes from observing the full spectrum of phenotypes while changing both ploidy and parental genome ratio.

Effects of altering either ploidy or parental genome ratio
Both 4x × 2x and 2x × 4x crosses produce 3x embryos, but seeds (and embryos) from the former cross are abnormally small at maturity, while those from the reciprocal cross are abnormally large. Embryos from the former cross contain double the normal dose of maternal genomes (2m:1p) while the latter contain a double paternal dose (1m:3p). (These embryos are also associated with endosperms of different constitution; this will be considered below.) Similarly, 4x × 4x, 6x × 2x, and 2x × 6x crosses are all predicted to produce 4x embryos. However, the 2m:2p embryos in the balanced 4x × 4x crosses are viable, while the 3m:1p and 1m:3p embryos produced by the interploidy crosses abort by heart stage.

Abnormal seed development is also correlated with altered parental genome dosage in the endosperm. 4x × 2x and 2x × 6x crosses both produce 5x endosperms. However, 4m:1p endosperms from 4x × 2x crosses show inhibited mitosis and support viable embryos, while 2m:3p endosperms from 2x × 6x crosses show accelerated mitosis, failure of cellularisation,
and dramatic overproliferation, and are invariably associated with embryo abortion.

In contrast to the above, embryos or endosperms with a constant ratio of parental genomes do not develop very differently when their ploidy is altered. This is illustrated by the series of balanced crosses. In these, the parental ratios in embryos and endosperms are constant (1m:1p and 2m:1p respectively), but the ploidies are increased (Table 1). Nevertheless, all of the balanced crosses produce viable embryos, and endosperms which show very similar developmental patterns (Figs 5, 8). In a background of constant embryo and endosperm parental ratios, the size of the mature seeds does increase with overall number of genomes, but altering parental genome ratio has an even greater effect on seed size (Fig. 2). Taken together, the evidence rules out genome dosage per se in the embryo or endosperm as a critical factor in seed development.

Effects of altering both ploidy and parental genome ratio
Varying the parental genome ratio in Arabidopsis seeds produces a spectrum of phenotypes in the embryo and endosperm, affecting characters such as seed viability, seed

![Fig. 5. For legend see p. 3337](image-url)
weight, rate of mitosis in the endosperm, and timing of endosperm cellularisation. These characters vary smoothly with the ratio of maternal to paternal genomes in the seed. For example, as ratios are manipulated from the maximum maternal excess generated in our experiments (a triple dose of maternal genomes relative to paternal genomes) through balanced ratios through the maximum paternal excess (a triple dose of paternal genomes), seeds move from inviable to viable back to inviable (Table 1; Fig. 1), seed weights as well as numbers of endosperm nuclei increase (Figs 2, 8), and endosperm cellularisation occurs later (Figs 5, 6). In contrast, the ploidy of the endosperm or embryo appears not to be correlated with the variation in the seed characters examined. For example, 3x and 6x (2m:1p) endosperms develop normally, but endosperms with an intermediate ploidy of 5x (4m:1p or 2m:3p) show markedly different phenotypes.

**Distinguishing effects of parental genome imbalance in the embryo and the endosperm**

The evidence above indicates that in *Arabidopsis* seeds the parental genome ratio, rather than genome dosage per se, is crucial for normal development. However, interploidy crosses...
simultaneously change the genome balance in the embryo and endosperm in the same direction, and consequently our data cannot directly establish whether the effects we see are due to imbalance in the embryo, or the endosperm, or both. Nevertheless, the seed phenotypes we observe following interploidy crosses suggest that altering the parental genome ratio in the seed has its major direct effect on the endosperm. In seeds with either maternal or paternal excess – even those that will abort due to a lethal triple dose – embryo development appears roughly normal (though slow when there is maternal excess). In aborting

**Fig. 5.** Embryo and endosperm development following balanced and interploidy crosses. Confocal micrographs of Feulgen-stained seeds 2-7 DAP. With the exception of 4 DAP and 7 DAP, each stage is represented by two views centred on the micropylar and chalazal poles (MP and CP) to illustrate embryo and chalazal endosperm development, respectively. Central peripheral endosperm (CPE) is shown in an additional column for 4 DAP, and in insets for 2x × 4x and 2x × 6x, 5 DAP. A single micrograph is provided for 7 DAP, in most cases showing the embryo. Crosses producing maternal excess (4x × 2x, 6x × 2x) are characterised by poor endosperm development, early cellularisation of the endosperm, and retarded embryogenesis, while crosses producing maternal excess (2x × 4x, 2x × 6x) result in overgrown endosperms and delay or failure of endosperm cellularisation. Scale bars, 25 μm.
seeds, the major phenotypic effect on the embryo appears to be its arrest. In contrast, endosperms with maternal or paternal excess show dramatic and reciprocal phenotypes.

Our conclusion that altered parental genome balance in the endosperm is primarily responsible for abnormal seed development is supported by experiments in maize where embryo and endosperm constitution were changed independently (Sarkar and Coe, 1966; Lin, 1984). These studies show that the genetic composition of the endosperm is critical for normal seed formation and viability, while the karyotype of the embryo (including its m:p ratio) appears to be far less important. Moreover, as the function of the endosperm in the early seed is believed to be transmission of nutrients and possibly growth regulators to the developing embryo (Brink and Cooper, 1947; Maheshwari, 1950; Bhatnagar and Sawhney, 1981; Vijayaraghavan and Prabhakar, 1984; Lopes and Larkins, 1993), normal endosperm function is expected to be of primary importance in seed development.

From our data alone we cannot rule out the possibility that the seed phenotypes we see are due to disturbed ploidy relationships between embryo, endosperm, and/or sporophytic tissues. However, Lin (1984) observed normal kernel formation on maize plants in which these ratios had been altered, and concluded that they are not critical for seed development.

**Resource allocation and parental imprinting during seed development in Arabidopsis**

**Role of endosperm in embryo nutrition**

We have argued above that the main direct effect of altering the parental genome ratio is on the endosperm rather than the embryo. Since there is little endosperm in a mature Arabidopsis seed, our evidence suggests that the viability and the final size of a seed reflect the effectiveness of the endosperm in provisioning the embryo during its development. At least three parameters of endosperm development or growth are disturbed following interploidy crosses in Arabidopsis: numbers of nuclei, timing of cellularisation, and development of particular regions of the endosperm. Similar effects of altered parental genome balance in the seed have been observed in other species, including brassicas (Håkansson, 1956; Nishiyama and Inomata, 1966) and rye (Håkansson and Ellerström, 1950).

In maize and wheat, endosperms with more cells accumulate more storage materials (Brocklehurst, 1977; Ouattar et al., 1987). Therefore, a large endosperm with many cells resulting from paternal genomic excess might accumulate more resources from the seed parent than a small endosperm resulting from maternal excess. Although endosperm and embryo size are abnormal following 4x × 2x and 2x × 4x crosses, the high seed viability in these crosses shows that the endosperm is still able to acquire maternal resources and transmit them to the embryo. However, the phenotypes of aborted seeds from 6x × 2x crosses suggest that lethal maternal excess prevents the endosperm from acquiring sufficient resources to sustain itself or the embryo; while the phenotypes of 2x × 6x seeds imply that endosperms with lethal paternal excess ultimately fail to accumulate and/or transmit maternal resources.

It is not clear how cellularisation of the endosperm affects embryo nutrition, particularly since timing of cellularisation is variable among different groups of angiosperms, and in many

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**Fig. 6.** Stages of embryo development and timing of peripheral endosperm cellularisation following balanced and interploidy crosses.

<table>
<thead>
<tr>
<th>2 DAP</th>
<th>3 DAP</th>
<th>4 DAP</th>
<th>5 DAP</th>
<th>6 DAP</th>
<th>7 DAP</th>
</tr>
</thead>
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<tr>
<td>6x-2x</td>
<td>zygote</td>
<td>zygote</td>
<td>globular</td>
<td>globular</td>
<td>globular</td>
</tr>
<tr>
<td>4x-2x</td>
<td>zygote</td>
<td>zygote to 2-terminal cell</td>
<td>globular to heart</td>
<td>globular</td>
<td>globular</td>
</tr>
<tr>
<td>2x-2x</td>
<td>zygote to dermatogen</td>
<td>globular</td>
<td>globular to heart</td>
<td>heart to torpedo</td>
<td>linear cotyledon</td>
</tr>
<tr>
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<td>zygote to 1-2-terminal cell</td>
<td>globular</td>
<td>globular</td>
<td>heart</td>
<td>linear cotyledon</td>
</tr>
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<td>globular</td>
<td>heart</td>
<td>torpedo</td>
</tr>
<tr>
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<td>globular</td>
<td>globular to heart</td>
<td>heart</td>
<td>heart to torpedo</td>
</tr>
<tr>
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<td>1-terminal cell</td>
<td>dermatogen to globular</td>
<td>globular to heart</td>
<td>globular to heart</td>
<td>heart</td>
</tr>
</tbody>
</table>

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**Fig. 7.** Development of tetraspore mutant seeds. Confocal micrographs of seeds from a single silique produced by a self-pollinated tes mutant. (A) Embryo and (B) chalazal endosperm from a single seed. The embryo is at the late heart stage, and the endosperm closely resembles that of seeds derived from balanced crosses at a similar stage (see Fig. 5, cross 2x × 2x, 5 and 6 DAP). (C) Embryo, (D) chalazal endosperm, and (E) central peripheral endosperm near the chalazal pole, from another single seed. In contrast to the seed shown in A and B, the embryo has only reached the globular/heart stage, and overgrown, vacuolate endosperm is filling the seed; these are characteristics of lethal paternal excess (see Fig. 5, cross 2x × 6x, 5-7 DAP). Scale bar, 25 μm.
sac and the vascular connection to the seed parent, implying chalazal proliferating tissue, which lies between the embryo (Yeung and Meinke, 1993). Chalazal endosperm appresses the suspensor, which in turn serves as the conduit to the embryo role in transferring nutrients and growth factors to the seed parent enter the embryo sac of dicots through the chalazal and micropylar poles (Håkansson, 1956; Schulz and Jensen, 1971, 1974; Mansfield et al., 1991; Chamberlin et al., 1993). An alternative explanation for our results is that there is a dosage-dependent relationship between (1) the total number of genomes in the primary endosperm nucleus, and (2) factors encoded by the maternal genome and deposited in the central cell cytoplasm before fertilisation: this was proposed by Birchler (1993) to account for the 2m:1p requirement in maize endosperm described by Lin (1984) without involving imprinting. However, the existence of dosage-dependent factors in the central cell cytoplasm remains hypothetical, while there is molecular evidence for functional non-equivalence of maternal and paternal genomes in endosperm (Lund et al., 1995). We believe that imprinting is the most likely explanation of our results.

According to the parental conflict model of Haig and colleagues (Haig and Westoby, 1989, 1991; Moore and Haig, 1991), imprinting evolved in both mammals and flowering plants as a consequence of conflict between the maternal and paternal genomes over resource allocation from the mother to the embryo. This is proposed to arise because the reproductive fitness of a mother is greatest when she distributes resources equally among all her offspring, while a father benefits when maternal resources are concentrated in his own offspring. In the case of plants, since endosperm mediates transfer of resources from seed parent to embryo, the model predicts that maternally and paternally derived alleles will be selected to have opposite effects on endosperm (and ultimately embryo) growth. Adding paternal genomes to the seed is expected to provide extra doses of the unparentally expressed alleles that increase seed size, while extra maternal genomes are predicted to provide an excess of alleles that limit seed size. Our results provide support for the parental conflict theory, as we find that Arabidopsis seeds with double the normal dose of paternal genomes produce large endosperms and embryos, while those containing a double dose of maternal genomes have opposite phenotype.

The evidence presented above suggests that Arabidopsis requires a 2m:1p ratio in the endosperm for normal seed growth. This implies that maternally and paternally derived genomes are not functionally equivalent in Arabidopsis endosperm, and therefore is consistent with evidence from other plant species that endosperm development requires activity of imprinted genes (Kermicle, 1970; Lin, 1982, 1984; Haig and Westoby, 1989, 1991; Kermicle and Alleman, 1990; Lund et al., 1995). An alternative explanation for our results is that there is a dosage-dependent relationship between (1) the total number of genomes in the primary endosperm nucleus, and (2) factors encoded by the maternal genome and deposited in the central cell cytoplasm before fertilisation: this was proposed by Birchler (1993) to account for the 2m:1p requirement in maize endosperm described by Lin (1984) without involving imprinting. However, the existence of dosage-dependent factors in the central cell cytoplasm remains hypothetical, while there is molecular evidence for functional non-equivalence of maternal and paternal genomes in endosperm (Lund et al., 1995). We believe that imprinting is the most likely explanation of our results.

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The parental conflict model proposes that selection for imprinting occurs when parents experience different reproductive costs. In Arabidopsis thaliana, mothers and fathers generally bear the same cost for each seed because they are the same individual, as even in the wild this species is almost completely self-fertilising (Abbott and Gomes, 1989). However, it is probable that A. thaliana, like other inbreeding plants, evolved from outcrossers (Stebbins, 1974). Therefore we suggest that A. thaliana retains a parental imprinting system inherited from outcrossing ancestors, which has partially broken down as
inbreeding has become predominant, allowing development of viable seeds with a limited degree of maternal or paternal excess.

The parental conflict model predicts that extra doses of paternally expressed loci will result in increased provisioning of the offspring via the endosperm, but this process appears to fail in seeds with lethal paternal excess. During the first few days of seed development in 2x × 6x crosses, both embryo and endosperm grow rapidly. Subsequently, the micropylar peripheral and chalazal endosperm and the chalazal nodules show dramatic overproliferation and eventually fill the seed – apparently at the expense of both the central peripheral endosperm, which begins to divide more slowly than in 2x × 4x crosses at 4 DAP (Fig. 8), and the embryo. This is not necessarily inconsistent with the prediction. Hurst and McVean (1997) argue that if changes in dosage of imprinted loci are sufficient to cause lethality, the phenotypes observed may not be a good indication of the role of those loci in normal development. These authors also propose an extension to the parental conflict model, suggesting that paternally derived alleles could be selected to extract resources from the mother but sequester them in the placenta (which could be argued to have a similar function to endosperm); thus paternal excess would produce a large endosperm but small embryo, as we observe in 2x × 6x crosses. However, since seeds with viable paternal excess have large embryos as well as large endosperms, it seems more likely that the lethal paternal excess phenotype is due to catastrophic failure of endosperm function.

Recently the MEDEA (MEA) gene has been identified as a candidate imprinted locus in Arabidopsis (Grossniklaus et al., 1998). Seeds inheriting mutant mea alleles through the mother contain large embryos (which abort at late heart stage) but small endosperms; the authors propose that the embryo undergoes increased cell proliferation at the expense of the endosperm. However, this does not necessarily contradict the hypothesis that imprinted genes function primarily in endosperm. MEA transcription begins in the female gametophyte before pollination, persisting after fertilisation until seed maturity. The authors conclude that MEA could be exclusively a maternal-effect gene; or it could be expressed both in the female gametophyte and in one or both fertilisation products, in which case imprinting could still explain only part of its regulation.

Unlike other model species, Arabidopsis can produce large quantities of abnormal but viable seeds with a double dose of paternal or maternal genomes. In addition, the small size and rapid development of Arabidopsis seeds make cytological investigation of early endosperm development less laborious than in maize. These attributes, coupled with the ease of conducting genetic screens and subsequent gene cloning in Arabidopsis, should enable identification and isolation of imprinted loci involved in seed development.

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