the plant journal

SEB Society for Experimental Biology

The Plant Journal (2012) **71**, 503–516 doi: 10.1111/j.1365-313X.2012.05015.x

Parental genome imbalance in *Brassica oleracea* causes asymmetric triploid block

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Received 3 August 2011; revised 19 March 2012; accepted 28 March 2012; published online 8 June 2012.

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SUMMARY

Interploidy crosses fail in many plant species due to abnormalities in endosperm development. In the inbreeding species *Arabidopsis thaliana*, both paternal and maternal excess interploidy crosses usually result in viable seed that exhibit parent-of-origin effects on endosperm development and final seed size. Paternal excess crosses result in extended proliferation of the endosperm and larger seeds, while conversely maternal excess crosses result in early endosperm cellularisation and smaller seeds. Investigations into the effect of parental gene dosage on seed development have revealed that MADS box transcription factors, particularly the AGAMOUS-like family, play important roles in controlling endosperm proliferation. The important crop genus *Brassica* contains self-incompatible outbreeding species and has a larger and more complex genome than the closely related Arabidopsis. Here we show that although *Brassica oleracea* displays strong parent-of-origin effects on seed development, triploid block due to lethal disruption of endosperm development was restricted to paternal excess, with maternal excess crosses yielding viable seed. In addition, transcriptome analyses of *Brassica* homologues of Arabidopsis genes linked to parent-of-origin effects revealed conservation of some mechanisms controlling aspects endosperm behaviour in the two species. However, there were also differences that may explain the failure of the paternal excess cross in *B. oleracea*.

Keywords: *Brassica*, interploidy crosses, seed development, endosperm, MADS box.

INTRODUCTION

Interploidy crosses play an important role in polyploid speciation (Köhler *et al.*, 2010) where post-zygotic seed lethality can result in genetically distinct populations of divergent ploidy. The consequences of interploidy crosses for seed development have been studied across a wide range of species: *Arabidopsis thaliana*, *Triticum* spp., *Zea mays*, *Secale cereale* and *Brassica* species (Watkins, 1932; Howard, 1939; Håkansson, 1952, 1956; Nishiyama and Inomata, 1966; Scott *et al.*, 1998). The outcomes of reciprocal crosses between different ploidy levels are known to vary considerably amongst different taxa, but are frequently lethal due to highly aberrant endosperm development (Watkins, 1932; Håkansson, 1956; Lin, 1984; Scott *et al.*, 1998). Among a variety of hypotheses put forward to explain these effects the best supported is 'parental genome dosage' in which the developmental abnormalities observed during seed development result from deviations from the normal two maternal:one paternal genome ratio in the endosperm (Lin, 1984).

The effects of interploidy crosses have been comprehensively examined in Arabidopsis (Scott *et al.*, 1998). The authors reported viable seed from both maternal excess ($4x \times 2x$; ♀–♂) and paternal excess ($2x \times 4x$; ♀–♂) crosses. This revealed a reduction in the severity of the response of Arabidopsis to interploidy crosses compared with previously examined species. Altering parental genome dosage affected endosperm proliferation (nuclear division and cellularisation), as previously observed for *Brassica* species (Nishiyama and Inomata, 1966) and *Z. mays* (Lin, 1984), but did not prevent differentiation of a functional endosperm and the production of viable seed. Significantly, this modest, non-lethal response to parental imbalance provides a route to modulating seed size that can contribute to increased seed yield in crops, including brassicas.

Brassicas are close relatives of Arabidopsis that diverged about 20 million years ago (Yang *et al.*, 1999), but generally display a severe, lethal response to even modest parental genomic imbalance. Although a few studies were carried out

in brassicas 45–70 years ago (summarised in Table 1), there were no common criteria for assessment of endosperm and seed development (Howard, 1939; Håkansson, 1956; Nishiyama and Inomata, 1966). Additionally, the plant material used for these experiments was poorly described, and was likely to be highly heterozygous due to strong self-incompatibility present in *Brassica oleracea*. Following pollinations between diploids and tetraploids in both *Brassica rapa* and *B. oleracea*, endosperm development was strongly disturbed, and although embryo development was normal in the early stages, it was eventually aborted (Håkansson, 1956). Endosperm development following maternal excess crosses within these species produced highly reduced endosperm, due to a reduced mitotic rate and premature cellularisation (Håkansson, 1956; Nishiyama and Inomata, 1966; Scott *et al.*, 1998). In contrast, paternal excess resulted in endosperm over-proliferation, with either delayed cellularisation in Arabidopsis $2x \times 4x$ seeds or no cellularisation in *Brassica* $2x \times 4x$ seeds and Arabidopsis $2x \times 6x$ seeds (Nishiyama and Inomata, 1966; Scott *et al.*, 1998). In Arabidopsis, where $4x \times 2x$ and $2x \times 4x$ seed is viable, the size of the endosperm is positively correlated with final seed size (Scott *et al.*, 1998).

The parent-of-origin effects observed in the endosperm following interploidy crosses have been attributed to genomic imprinting within the gametes (Adams *et al.*, 2000; Vinkenoog *et al.*, 2000; Erilova *et al.*, 2009). Genomic imprinting occurs in flowering plants and mammals, and involves the gender-specific silencing of alleles of genes (Scott and Spielman, 2006; Feil and Berger, 2007). Functional analysis of imprinted genes in mammals shows that maternally silent genes tend to encode growth promoters while paternally silent genes encode growth inhibitors (Tycko and Morison, 2002). This provides strong support for the proposition that parent-of-origin effects resulting from genomic imbalance are due to the operation of imprinting (Haig and Westoby, 1989), since an extra dose of a maternal genome provides an excess of active growth inhibitor alleles, and vice versa for paternal excess.

A number of imprinted genes, including members of the *FERTILISATION INDEPENDENT SEED* (*FIS*) Polycomb Group (PcG) complex, have been identified in Arabidopsis (Chaudhury *et al.*, 1997; Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999), and again functional analysis points to imprinting as the cause of parent-of-origin effects in endosperm development (Dilkes *et al.*, 2008; Erilova *et al.*, 2009). In the Columbia ecotype of Arabidopsis, $2x \times 4x$ crosses result in lethal endosperm over-proliferation similar to $2x \times 6x$ crosses in the ecotypes L*er* and C24 (Redei, 1964; Dilkes *et al.*, 2008). Erilova *et al.* (2009) provide direct evidence that imprinting of *MEA* is the principal cause of endosperm failure in Columbia $2x \times 4x$ seeds. These authors argue that since *MEA* is maternally expressed in the endosperm, the relative level of repressive FIS PcG complex provided by the maternal genome is diluted in a paternal excess endosperm leading to the de-repression of genes normally held in check by the PcG complex, such as *PHERES 1* (*PHE*), a maternally silent potential growth promoter (Köhler *et al.*, 2005) and *AGL62*, a potent repressor of endosperm cellularisation (Kang *et al.*, 2008). Significantly, effects of paternal excess are reduced by over-expression of *MEA* in $2x \times 4x$ endosperm. The relative abundance of FIS PcG complex in $4x \times 2x$ seeds may explain the maternal excess phenotype.

In this paper we set out to re-examine the behaviour of interploidy crosses in *B. oleracea* (henceforth referred to as *Brassica*) using improved genetic material and high-throughput sequencing platforms. We undertook a detailed characterisation of the effects of interploidy crosses in *Brassica* for the first time using isogenic genetic material to provide a systematic and exhaustive comparison with

Table 1 A comparison of the datasets obtained by this and previous studies of interploidy crosses in *Brassica* species

Species	Reference	Germination rate	Seed weight/size	Description of progeny	Quality of plant material	Developmental description	Gene expression examined
B. oleracea	Howard (1939)	n.d.	Seed weight for 10 seed from each cross reported	Ploidy tested	Likely to be highly heterozygous	n.d.	No
B. oleracea and *B. rapa*	Håkansson (1956)	n.d.	n.d.	n.d.	Likely to be highly heterozygous	Pictures taken of seeds between 1 and 21 DAP	No
B. rapa	Nishiyama and Inomata (1966)	n.d.	n.d.	Ploidy tested, morphological descriptions	Likely to be highly heterozygous as used two different species	Drawings from seeds sectioned between 1 and 21 DAP	No
B. oleracea	This paper	Yes	Seed size and weight recorded	Ploidy tested, morphological descriptions	Highly homozygous	High-quality images from 5 to 25 DAP	Yes

n.d., not determined; DAP, days after pollination.

studies in Arabidopsis. In previous studies in brassicas, the diploid and tetraploid parents were genetically distinct, which necessarily complicated interpretation of the phenotype, since both interspecific and interploidy crosses cause endosperm effects (Haig and Westoby, 1991; Ishikawa and Kinoshita, 2009). This experimental system also gave us the opportunity to identify any conserved mechanisms between the model species Arabidopsis and *Brassica*. Our aim is to understand the mechanism which could provide novel routes to crop improvement through the manipulation of endosperm development via genomic imprinting.

RESULTS

Brassica demonstrates an asymmetric triploid block

Three sets of cross-pollinations using diploid and tetraploid *Brassica* lines were performed to determine the effect of parental genome dosage on seed development. Pollination was carried out manually for both self- and cross-pollination. At maturity, siliques were harvested and analysed for seed set and size (Figure 1a–d). In addition, seeds from reciprocal interploidy crosses ($2x \times 4x$ and $4x \times 2x$) were germinated to test for viability and ploidy analysis. The actual and predicted outcomes from the balanced and parental excess crosses are detailed in Table 2.

There was a dramatic difference in the frequency of fertilised ovules between the diploid and tetraploid parents. The balanced $2x \times 2x$ crosses produced an average of 19.4 seeds per silique, whereas the $2x \times 4x$ cross yielded an average of 3.4 seeds per silique, all of which failed to develop to maturity (Table 2). Similarly, the $4x \times 4x$ and $4x \times 2x$ crosses produced very few seeds, indicating that both male and female gamete production in tetraploids is compromised.

In all our experiments, the $4x \times 2x$ maternal excess cross produced smaller and lighter seeds (50 and 27%, respectively) compared with the $2x \times 2x$ cross (Figure 1m,n), which germinated at a high frequency of 90% (Table 2), a rate comparable to the control $2x \times 2x$ and $4x \times 4x$ seeds. Ploidy analysis confirmed that the majority of these seedlings were triploid (Table 2). These triploid plants grew normally, resembling either $2x$ or $4x$ plants in phenotype, but failed to set seed.

In contrast to the outcome of the maternal excess cross, paternal excess crosses produced shrivelled seeds (Figure 1d) that failed to germinate (Table 2). This is in contrast to the situation observed in Arabidopsis ecotypes that have

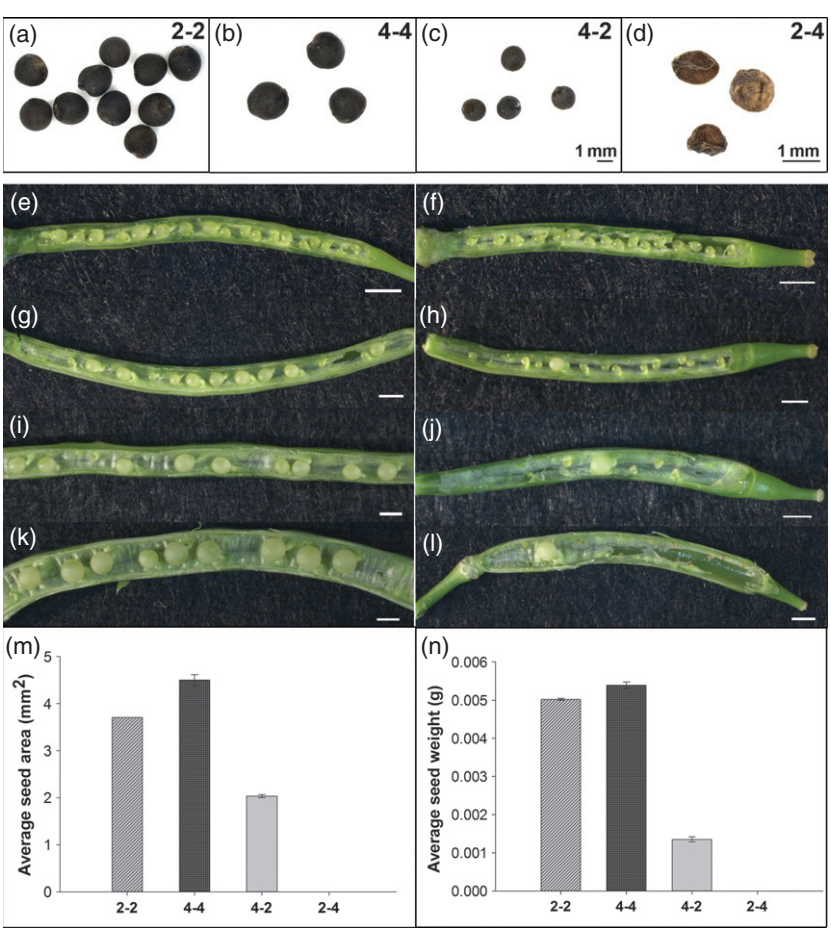

Figure 1. Seed development following balanced and interploidy crosses.
Panels (a–d) show mature seed obtained from single siliques from balanced $2x \times 2x$ (a), $4x \times 4x$ (b) and interploidy $4x \times 2x$ (c), $2x \times 4x$ (d) crosses.
Photographs of single siliques cut open longitudinally from balanced $2x \times 2x$ crosses 7, 10, 16 and 22 days after pollination (DAP) are shown in panels (e–h), respectively. The corresponding siliques from a $2x \times 4x$ cross at the same DAP are shown in panels (i–l). The smaller seed observed in the siliques in panels (j) and (k) ($2x \times 4x$, 10, 16 DAP) are unfertilised ovules which can be observed as small dark aborted ovules in the 22-DAP silique (l).
The average size of the $4x \times 4x$ seeds was approximately 15% larger than the $2x \times 2x$ seeds, whereas the $4x \times 2x$ seeds are almost 50% smaller (m). This difference is reflected in the weight of the seed with $4x \times 2x$ seeds on average weighing a quarter of the weight of mature $2x \times 2x$ seeds weight (n).
Bar = 1 mm for (a–d) and 2 mm for (e–l).

Table 2 Predicted and actual outcomes from balanced and parental excess crosses

Cross	Seed parent	Pollen parent	Predicted embryo ploidy (m:p)	Predicted endosperm ploidy (m:p)	Mean no. of viable seeds/silique ± SE (no. of siliques)	% Seed germinated (no. of seeds tested)	Seedling ploidy (no. of seedlings tested)
$2x \times 2x$	$2x$	$4x$	$2x$ (1:1)	$3x$ (2:1)	19.4 ± 0.35 (25)	100 (20)	$2x$ (4)
$4x \times 4x$	$4x$	$4x$	$4x$ (2:2)	$6x$ (4:2)	2.6 ± 0.19 (3)	80 (20)	$4x$ (4)
$4x \times 2x$	$4x$	$2x$	$3x$ (2:1)	$5x$ (4:1)	1.8 ± 0.12 (10)	90 (20)	$3x$ (14) $6x$ (2)
$2x \times 4x$	$2x$	$4x$	$3x$ (1:2)	$4x$ (2:2)	0[a]; 3.4 ± 0.31 (68)[b]	0[a]	0[a]

m, maternal; p, paternal.
[a]One triploid and one tetraploid seedling were obtained from the paternal excess cross, but most seed failed to develop.
[b]Number of seed per silique that were successfully fertilised, but subsequently failed to develop.

been examined where fertility following $2x \times 4x$ crosses only decreases by 30% when compared with a balanced $2x \times 2x$ cross (Scott *et al.*, 1998). To understand the reason for seed failure, we first used seed clearing to examine seeds across a broad developmental window 7–22 days after pollination (DAP). At 7 DAP, the siliques from $2x \times 4x$ crosses were similar in appearance to $2x \times 2x$ siliques (Figure 1e,f). The first differences between the $2x \times 2x$ and $2x \times 4x$ crosses were observed at 16 DAP (Figure 1i–l). Fertilised $2x \times 4x$ seeds were paler than $2x \times 2x$ seeds and had ceased to expand. The embryo in the $2x \times 4x$ seeds was at the globular stage whilst the embryo in the $2x \times 2x$ seeds had progressed to the heart stage (data not shown). At 22 DAP, the embryo in the $2x \times 4x$ seeds appeared arrested at the early heart stage while the $2x \times 2x$ embryos had reached the 'walking stick' stage (data not shown).

In summary, in paternal excess crosses, fertilisation was successful, albeit at a low frequency. The success of maternal excess crosses and the failure of the paternal excess crosses indicated that *Brassica* displays a paternal-specific asymmetric triploid block similar to the Columbia ecotype of Arabidopsis. To investigate the underlying reasons for this behaviour, we fixed developing seeds from $2x \times 2x$, $4x \times 4x$, $2x \times 4x$ and $4x \times 2x$ crosses for detailed comparative developmental analyses.

Parental genome imbalance result in reciprocal endosperm development phenotypes

To assess the impact of parental genome imbalance on seed and endosperm development in *Brassica* we made sections of seeds harvested at 13, 16 and 19 DAP from reciprocal interploidy and balanced crosses. Figure 2 shows cross-sections of whole seeds assembled by overlapping a series of images of individual seeds captured at high magnification. The assembled images have been false-coloured to aid interpretation (embryo in green and chalazal endosperm in purple). Measurements taken from these and other sections were aggregated to provide quantitative data on embryo sac size as represented by the maximum cross-sectional area (Figure 3a), the progress of endosperm cellularisation as represented by the ratio of cellularised to uncellularised peripheral endosperm (Figure 3c), and chalazal endosperm size as represented by the maximum cross-sectional area (Figures 3b and S1). The size of the uncellularised peripheral endosperm at 13 DAP was also assessed using maximum area measurements (Figure 3d).

Balanced crosses. By 13 DAP in $2x \times 2x$ seeds the embryo was at the globular stage (Figure 2a). The suspensor was embedded in micropylar peripheral endosperm, uncellularised central peripheral endosperm lined the embryo sac, and the chalazal endosperm was prominent and multinucleate. By 16 DAP the embryo reached the early torpedo stage and cellularisation of the peripheral endosperm was well under way. The chalazal endosperm remained prominent. In seeds at 19 DAP the embryo had grown to the walking stick stage and the wave of endosperm cellularisation had progressed further toward the centre of the embryo sac. The cross-sectional area, and by extrapolation the volume of the seeds, increased markedly from 13–19 DAP. In the $4x \times 4x$ cross, the rate of embryo and endosperm development was slower than in the $2x \times 2x$ cross. Endosperm cellularisation was well under way at 16 DAP when the embryo was at the heart stage and the appearance of the chalazal endosperm throughout was similar to that in the $2x \times 2x$ seeds.

The quantitative measurements shown in Figure 3 reflect the above observations. Embryo sac area (Figure 3a) in $2x \times 2x$ and $4x \times 4x$ seeds increased in line with increases in seed area (Figure 2). Similarly, measurements of endosperm cellularisation showed that the majority of 13 DAP seeds had uncellularised endosperm, but by 19 DAP a significant proportion of the embryo sac contained cellularised endosperm (Figure 3c). The amount of uncellularised endosperm within the $2x \times 2x$ and $4x \times 4x$ seeds was similar (Figure 3d). In $2x \times 2x$ and $4x \times 4x$ seeds the chalazal endosperm attained near maximum size by 13 DAP (globular stage) and remained about the same size up to 19 DAP (walking stick stage in $2x \times 2x$ seeds) (Figure 3b).

Interploidy crosses. The development of *Brassica* maternal parental excess ($4x \times 2x$) seeds followed essentially the

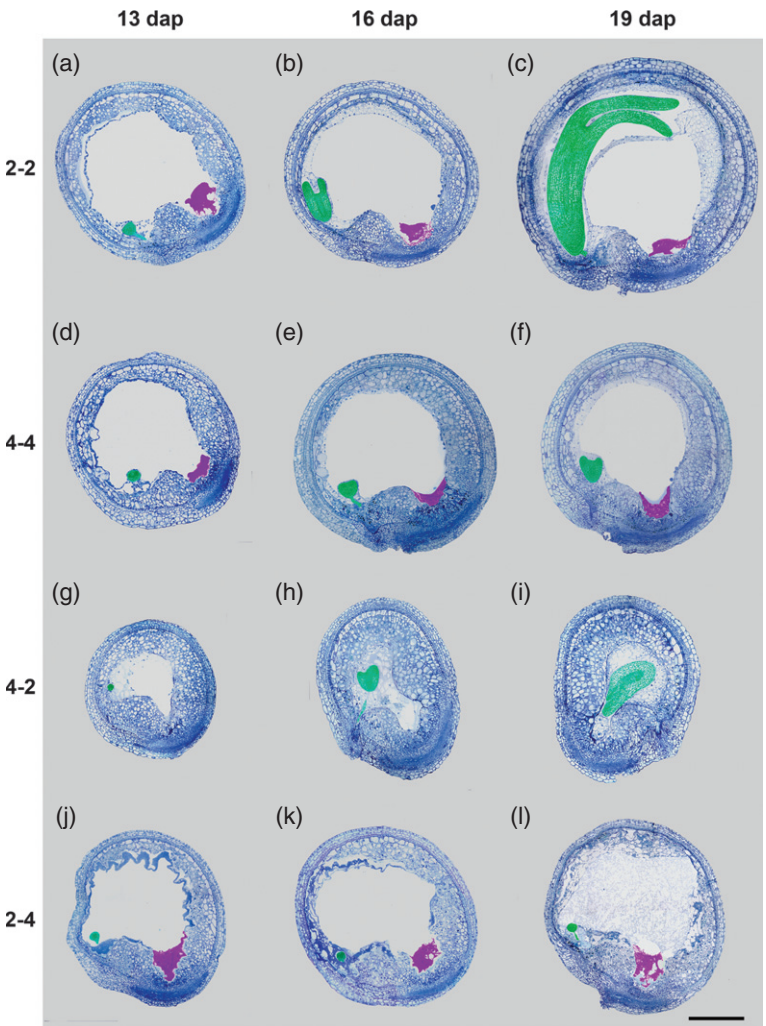

Figure 2. Embryo and endosperm development following balanced and interploidy crosses.
Light micrographs of stained sections of seed fixed at 13 (first column), 16 (second column) and 19 (third column) days after pollination (DAP). The embryo has been pseudo-coloured in green, the chalazal endosperm in purple for clarity.
Panels (a–c) are from a balanced cross $2x \times 2x$, (d–f) from $4x \times 4x$ while (g–i) are sections from seed produced from interploidy cross $2x \times 4x$ and (j–l) from $4x \times 2x$. Bar = 200 μm.

same pattern as reported previously for Arabidopsis. At 13 DAP the embryo was globular (Figure 2g–i) and contained a relatively small embryo sac. The endosperm showed signs of early cellularisation, and the chalazal endosperm was inconspicuous. At 16 DAP the embryo was at the heart stage. In contrast, the seed coat appears enlarged and to partially engulf the embryo. At 19 DAP, seed volume remained unchanged. The endosperm was fully cellularised and the embryo was at the walking stick stage, but was considerably smaller than embryos of the same developmental stage in $2x \times 2x$ seeds, and had abnormally short cotyledons. Paternal excess ($2x \times 4x$) seeds again shared features with seeds generated by modest and extreme paternal excess in Arabidopsis. However, there were significant differences; most notably that maximum seed volume was substantially smaller than for the balanced crosses and embryo development was very limited.

At 13 DAP the embryo was globular (Figure 2), and was contained in an embryo sac about the same size as in the balanced crosses. The peripheral endosperm was uncellularised and appeared over-proliferated relative to that in a balanced cross. The chalazal endosperm also appeared much larger than in the balanced crosses. At 16 DAP seeds had undergone some expansion. At 19 DAP the seed volume was unchanged and embryo remained at the globular stage. The chalazal endosperm appeared fragmented. A peripheral endosperm was no longer apparent and the embryo sac contained particulate material that could represent degraded endosperm.

Quantitative measurements (Figure 3) support the above observations. The embryo sac area (Figure 3a) of $4x \times 2x$ seeds was essentially unchanged between 13 and 19 DAP, and at 19 DAP was approximately 15% that of $2x \times 2x$ seeds. In $2x \times 4x$ seeds the embryo sac area at 13 and 16 DAP was similar to that of the balanced crosses, but did not increase further, such that by 19 DAP its area was approximately 60% that of $2x \times 2x$ seeds. This contrasts with Arabidopsis where paternal excess crosses resulted in a relatively large seed volume irrespective of whether the seed was viable ($2x \times 4x$) or failed ($2x \times 6x$) (Scott *et al.*, 1998). The ratio of cellularisation (Figure 3c) showed that endosperm cellularisation was under way in $4x \times 2x$ seeds at 13 DAP, whereas seeds

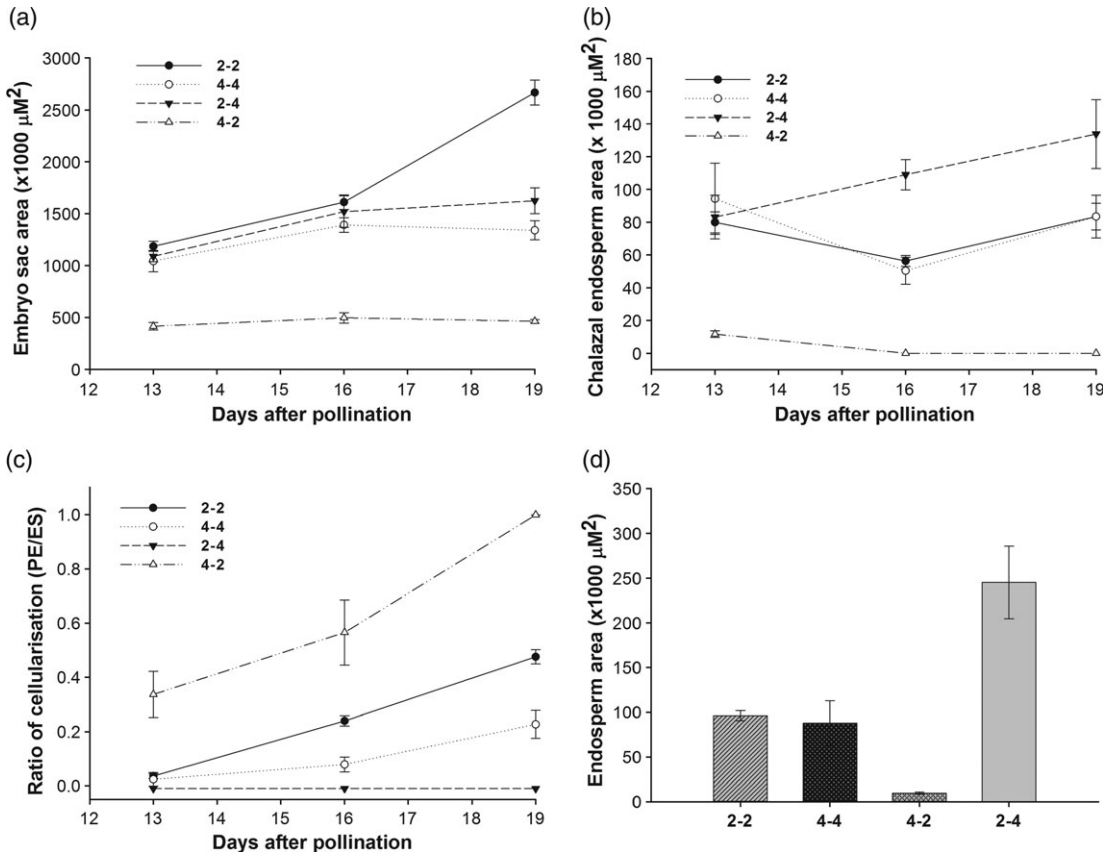

Figure 3. Graphs reporting quantitative measurements taken from assembled montages of fixed and sectioned seed from balanced and interploidy crosses at 13, 16 and 19 days after pollination (DAP).
Panel (a) shows embryo sac area measured as the maximum cross-sectional area (n = 4–26).
Panel (b) represents the chalazal endosperm area as maximal cross-sectional area (n = 3–24).
Graph (c) indicates the progress of endosperm cellularisation as represented by the ratio of cellularised to uncellularised peripheral endosperm (n = 3–25). The total endosperm area at 13 DAP as an average for each cross is represented in panel (d) (n = 3–12).

from balanced crosses at the same stage were completely uncellularised. This indicates that cellularisation initiates early in $4x \times 2x$ seeds. In contrast, no cellular endosperm was observed in $2x \times 4x$ seeds at the three time points investigated, suggesting that cellularisation is not initiated.

To determine whether interploidy crosses in *Brassica* result in variation in the amount of endosperm we measured the amount of uncellularised endosperm in seeds at 13 DAP (Figure 3d). At this time point, seeds from the two balanced crosses contained approximately the same amount of endosperm, whilst $4x \times 2x$ and $2x \times 4x$ seeds contain dramatically less endosperm – a 10-fold and 2.5-fold reduction, respectively. This indicates that parental excess in both Arabidopsis and *Brassica* have similar effects on endosperm proliferation during the uncellularised phase.

Paternal excess in *Brassica* does not cause seed over-expansion

The results described above reveal a significant difference in the behaviour of the endosperm generated by paternal excess in Arabidopsis and *Brassica*. The appearance of endosperm in $2x \times 4x$ seeds was strikingly different from that in the other crosses (Figure 4). At 13 DAP there was substantial free nuclear endosperm in the $2x \times 4x$ cross, but this appeared much less organised than endosperm in the other crosses. At 16 DAP, the $2x \times 4x$ endosperm had increased slightly in volume, but appeared highly abnormal relative to the other crosses, containing numerous vacuoles and a darkly staining cytoplasm. By 19 DAP the endosperm in $2x \times 4x$ seeds had undergone complete degeneration, whilst the endosperm of other crosses continued to develop normally.

In $2x \times 4x$ seeds, expansion of the embryo sac stops at 16 DAP (Figures 2 and 3a), coincident with the onset of endosperm degeneration (Figure 4). This suggests that the endosperm may provide the motive force to increase seed volume, which would also require expansion of the seed coat. In *Brassica*, the palisade cells in the outer integument of the seed coat had developed thick-walled cells by 16–19 DAP (Figure 4) in both balanced and interploidy

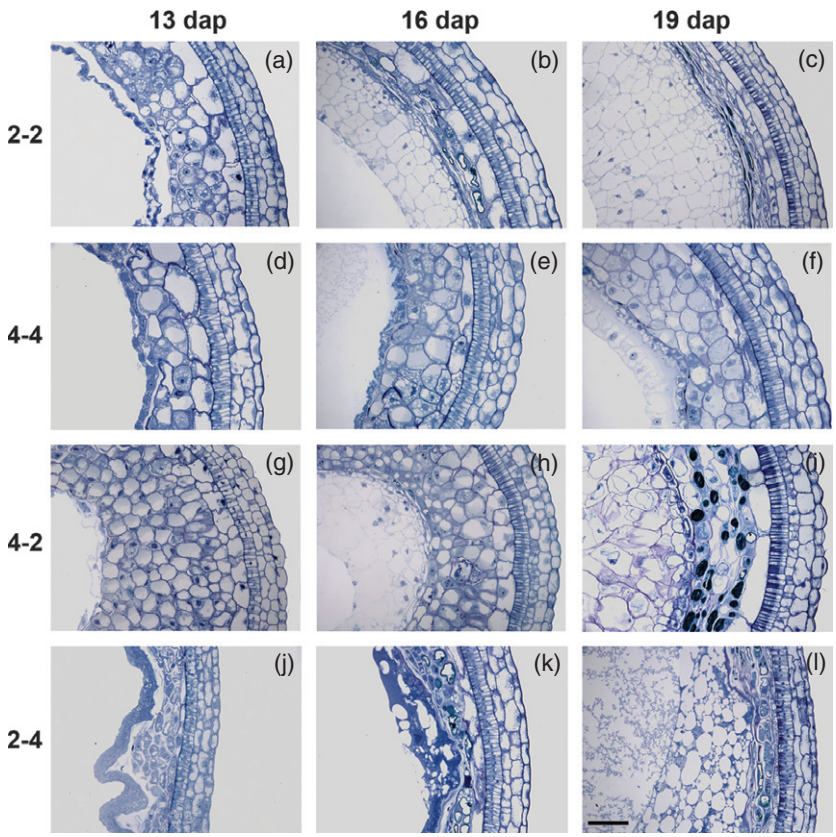

Figure 4. Development of the seed coat and peripheral endosperm following balanced and interploidy crosses.
Light micrographs of stained sections of seed fixed at 13 (first column), 16 (second column) and 19 (third column) days after pollination (DAP).
Panels (a–c) are from a balanced cross $2x \times 2x$, (d–f) from $4x \times 4x$ while panels (g–i) are sections of seed produced from interploidy cross $2x \times 4x$ and panels (j–l) from $4x \times 2x$.
Bar = 100 μm.

crosses as reported for *B. napus* seed coat (Nesi *et al.*, 2009). Although we do not have direct evidence, it is possible that a thick-walled layer in *Brassica* seed coats imposes greater resistance to seed expansion than in species like Arabidopsis, which lack this layer.

Embryo development ceases early in *Brassica* paternal excess seed

In Arabidopsis abortive crosses, endosperm cellularisation was highly precocious ($6x \times 2x$) or failed to occur ($2x \times 6x$). Despite these dramatic endosperm effects, the embryo reached the heart stage before seed development failed, although maternal excess slowed progression. In *Brassica*, the interploidy crosses had similar effects on endosperm development, with maternal excess promoting early cellularisation and paternal excess resulting in a failure of cellularisation (Figure 2, Table 3). In contrast to Arabidopsis, which demonstrated a 70% seed set rate from $2x \times 4x$ crosses, embryo development in the *Brassica* $2x \times 4x$ cross failed at the early heart stage as evidenced by a lack of viable seeds, indicating greater sensitivity to paternal excess.

Control mechanisms regulating the development of the endosperm in $2x \times 4x$ seeds

Evidence from previous studies indicates that the failure of endosperm development subsequent to interspecific and/or interploidy crosses results from abnormal development of the endosperm (Cooper and Brink, 1940; Scott *et al.*, 1998). The primary characteristic of the endosperm following an interploidy cross is an alteration in the timing of cellularisation, which is either advanced (maternal excess) or delayed (paternal excess) (Scott *et al.*, 1998). Kang *et al.*

Table 3 Stages of embryo development and timing of peripheral endosperm cellularisation following balanced and interploidy crosses (shading indicates level of cellularisation)

	10 DAP	13 DAP	15 DAP	16 DAP	19 DAP	20 DAP	25 DAP
$2x \times 2x$	Globular	Late globular	Heart	Late heart, torpedo	Early walking stick	Walking stick	Bent cotyledon
$4x \times 4x$	Globular	Globular	Heart	Heart	Heart	Heart	Walking stick
$4x \times 2x$	Globular	Late globular	Heart	Heart	Bent cotyledon	Bent cotyledon	Bent cotyledon
$2x \times 4x$	Globular	Globular	Globular	Globular	Globular	Globular	n.d.

DAP, days after pollination; n.d., not determined.

(2008) demonstrated in Arabidopsis that AGL62, a Type 1 MADS domain protein, is an important regulator of cellularisation during endosperm development. Aberrant expression of *AGL62* was confirmed by Tiwari *et al.* (2010) in a detailed study of transcriptional profiles associated with interploidy crosses. Genes strongly associated with enhanced or inhibited seed growth included a set of co-regulated MADS-box genes, *PHERES1*, *-2* (*PHE1*, *-2*), *AGL28*, *-40* (co-expressed with *PHE1*), *AGL62* (AGL62 interacts with PHE1), *AGL45* (AGL45 interacts with AGL40), and genes involved in hormone pathways and cell-cycle genes. To determine whether this network of genes exerted a similar level of control on endosperm cellularisation in *Brassica*, we investigated the expression of relevant genes associated with maternal and paternal excess (Tiwari *et al.*, 2010) using quantitative PCR. Additionally whole transcriptome analysis was carried out using RNA-seq on 16-DAP seed from $2x \times 2x$ and $2x \times 4x$ crosses.

Screening of existing *Brassica* expressed sequence tag (EST) and genome databases (including www.brassicadb.org), for homologues of genes encoding AGL62, -28, -40, -45 and PHERES1 and -2 revealed marked differences compared with Arabidopsis. To date, no genes with close homology to *PHERES1* or *-2* have been annotated in the genome. Sequences with high homology to *AGL62* and *AGL45* were identified but none to *AGL28* or *-40* (Table S1).

Quantitative PCR using RNA from seeds at 13, 16 and 19 DAP (Figure 5, Tables S2 and S3) was performed. Consistent with the pattern found in Arabidopsis (Kang *et al.*, 2008), expression of *AGL62* exhibited a decline in transcript levels in the balanced crosses which was correlated with cellularisation of the endosperm (Figure 5a). The level of *BolC.AGL62* expression in the maternal excess was low compared with the balanced crosses, as found in Arabidopsis. In contrast, the levels of *BolC.AGL62* did not show a decrease in the $2x \times 4x$ cross. Similar up-regulation of *AGL62* expression was observed in Arabidopsis $2x \times 4x$ and $2x \times 6x$ crosses (Tiwari *et al.*, 2010) and may be responsible for delayed or absent cellularisation.

The expression levels of another MADS box gene, *BolC.AGL45*, also up-regulated in paternal excess crosses in Arabidopsis, followed the same pattern as *BolC.AGL62*. Expression of *BolC.AGL45* fell dramatically during cellularisation of the endosperm in the balanced crosses (Figure 5b). The transcript level remained low in $4x \times 2x$ crosses, even at 13 DAP at which time-point the endosperm was completely cellularised. Again, the $2x \times 4x$ cross displayed higher levels of *BolC.AGL45* expression compared with balanced crosses.

Transcript analysis also demonstrated up-regulation of genes associated with proliferation of the endosperm (Tables 3 and S5). Two additional *AGL62* orthologues as well as *WRKY10* were found to be significantly up-regulated in the paternal excess cross. This up-regulation is also seen

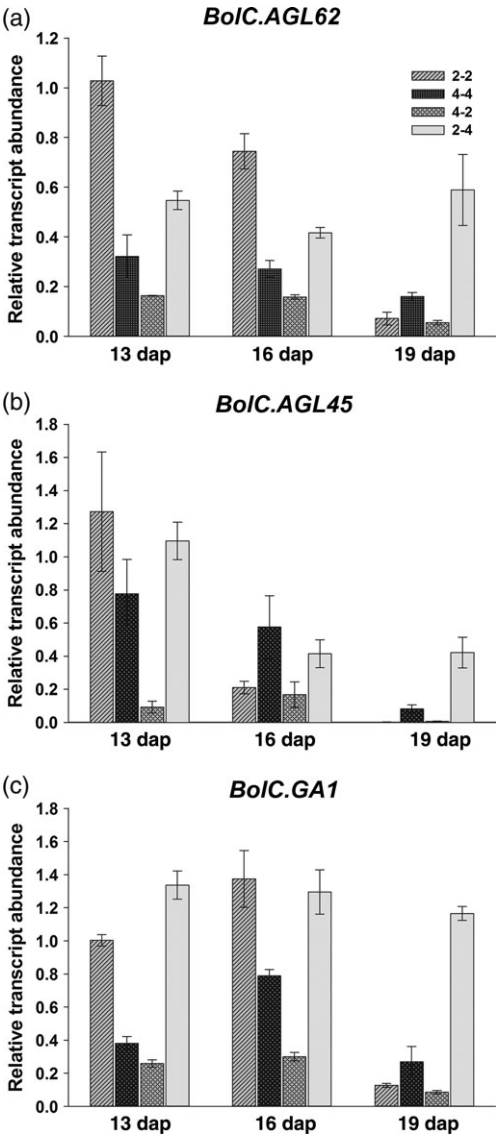

Figure 5. Relative transcript abundance of *BolC.AGL62* (*Bra038225*), *BolC.AGL45* (*Bra039404*) and *BolC.GA1* (*Bra036239*) from balanced and interploidy crosses at 13, 16 and 19 days after pollination (DAP).
Panels (a–c) show the relative transcript abundance of *BolC.AGL62*, *BolC.AGL45* and *BolC.GA1*, respectively, relative to *BolC.GAPC* (*Bra016729*). Three biological replicates were used for each cross at each time point except where otherwise noted. Error bars are standard error. *Two biological replicates used.

in Arabidopsis, suggesting an at least partially conserved mechanism controlling endosperm cellularisation.

Genes involved in gibberellin (GA) metabolism including *GA1* (At4g02780) were up-regulated in paternal excess crosses in Arabidopsis (Tiwari *et al.*, 2010). We have also found that transcription of the *Brassica* homologue *BolC.GA1* is up-regulated in the $2x \times 4x$ cross. The level of expression decreased two-fold in the balanced $2x$ crosses, although no change was observed in the corresponding $2x \times 4x$ cross (Figure 5c).

The comparison of the two transcriptomes revealed several other differentially expressed genes (Tables 4 and S5–S16). Of the 17 433 gene models identified, 577 were found to be significantly differentially expressed between the two transcriptomes, 167 up and 409 down ($P \leq 0.05$) (Table S5 and S6). Differential regulation was found for genes involved in endosperm development, cell-cycle regulation, cell growth, imprinting and chromatin and histone modification (Tables 4 and S7–S16).

Many maternally and paternally expressed genes (MEGS and PEGS) have recently been identified in Arabidopsis (Hsieh *et al.*, 2011; Wolff *et al.*, 2011). The developmental behaviour of seed generated by paternal excess in Arabidopsis could be explained by the global deregulation of imprinted genes resulting from the failure of the FIS PcG complex to control its target genes in imbalanced endosperms (Jullien and Berger, 2010). In support, Wolff *et al.* (2011) found that most MEGs and PEGs that were deregulated in an Arabidopsis *fis2* mutant were also significantly deregulated in triploid seeds derived from $2x \times 4x$ interploidy crosses. Since $2x \times 4x$ crosses in *B. oleracea* resulted in a paternal excess seed phenotype with features of extreme paternal excess in Arabidopsis, we reasoned that a similar deregulation of MEGs and PEGs in *B. oleracea* triploid seeds would both explain the seed phenotype and indicate a role for a FIS complex in regulating imprinted gene expression in this species. We compared the differentially expressed genes in *Brassica* paternal excess to the known MEGs and PEGs from Arabidopsis.

We found 264 genes (64.5%) in common with the list of maternally expressed genes from Wolff *et al.* (2011). Of these, 178 were down-regulated and 86 were up-regulated (Table S17). If the differential expression of the seed phenotype was due to imprinting it might be expected that in paternal excess, paternally expressed genes would be up-regulated and maternally expressed genes would be down-regulated. Consistent with these observations was the down-regulation in *Brassica* paternal excess of *AGO9*, which

Table 4 Selected differentially expressed genes from transcriptome analysis

Term/gene family	No. of *Brassica* gene models with significant differential expression	Most similar Arabidopsis genes	Expression in Tiwari *et al.* (2010)[a]	Explanation/conclusion
Agamous	Three up	*AGL62*[b]	Up in $2x \times 6x$	Expression of *AGL62* homologues is consistent with what is observed in Arabidopsis
		AGL12	Up in $6x \times 2x$	
	Five down	*AGL96*[b]	–	Expression of *AGL12*, *AGL40* and *AGL46* homologues is not consistent with what is observed in Arabidopsis. This may be due to genuine differential expression of these genes; however, it is not currently possible to assign these *Brassica* gene models as functional orthologues of Arabidopsis genes, especially given the reported rapid evolution of MADS1 genes (Nam *et al.*, 2004) and these *Brassica* genes may not be functional orthologues of the Arabidopsis genes
		AGL40	Up in $2x \times 4x$, $2x \times 6x$ Up in fis1 $\times$ 2*x*, Down in $6x \times 2x$	
		AGL46	Up in *fis1* $\times$ 2*x*	
		AGL67	–	
Endosperm	Three up	*AGL62*[b]	Up in $2x \times 6x$ and *fis1* $\times$ 2*x*	The over-expression of these genes consistent with what is seen in Arabidopsis seeds which fail to cellularise
		WRKY10	Up in $2x \times 6x$ and *fis1* $\times$ 2*x*	
Cell cycle	One down	*ATCUL1*	–	Expression of *DWF4* is consistent with what is observed in Arabidopsis as the *fis1* $\times$ 2*x* cross imitates a paternal excess phenotype and if gene expression is parentally biased, genes up-regulated in paternal excess should be down-regulated in maternal excess
Cell growth	Three up	*POM1*	–	
		DWF4	Up in *fis1* $\times$ 2*x*, Down in $4x \times 2x$	
		ATGH9A1	–	
	Three down	*CPD*	–	
		CUT1	–	
		PERX34	Up in $6x \times 2x$	
Chromatin modification	Two down	*VRN5*	–	Methylation and chromatin and histone modification are associated with imprinting, and changes in expression of genes might be anticipated in paternal excess cross. These are genes which are involved with imprinting which have not been reported previously in interploidy crosses
		PKL	–	
Histone modification	One down	*AT2G16485*	–	
Imprinting	Two down	*EMF2*	–	
		DME	–	
Methylation	One down	*DME*	–	

[a]Genes from consensus of the two array platforms used.
[b]Two *Brassica* models match *AGL62* and *AGL96*.

both Wolff *et al.* (2011) and Hsieh *et al.* (2011) characterised as a MEG in Arabidopsis. Another MEG encoding *AGL96* was also down-regulated (Table S17). To our surprise, no PEGs matched the up-regulated genes in our *Brassica* dataset (Table S17) further strengthening the idea that other mechanisms in addition to down-regulation of FIS complexes may play a role in the behaviour of endosperm produced by interploidy crosses.

DISCUSSION

In this paper we describe the outcomes of interploidy crosses in *Brassica*. We found that $4x \times 2x$ maternal excess crosses consistently produced small but viable seeds. Howard (1939) similarly reported that $4x \times 2x$ crosses in *Brassica* produced small triploid seeds that germinated at high frequency. However, two subsequent studies in brassicas reported little success in obtaining viable seeds from either $4x \times 2x$ or $2x \times 4x$ interploidy crosses (Håkansson, 1956; Nishiyama and Inomata, 1966). Consistent with these reports we found that $2x \times 4x$ crosses failed. We therefore performed a comprehensive analysis of endosperm and seed development following genome imbalance resulting from interploidy crosses in *Brassica*, including transcriptome sequencing from $2x \times 2x$ and $2x \times 4x$ seeds and quantitative PCR from balanced and interploidy seeds.

Parent-of-origin effects in *Brassica*

The data presented in this paper clearly demonstrate that *Brassica* displays strong parent-of-origin effects on seed development and size as a result of crosses in either direction between isogenic diploid and tetraploid parents. However, triploid block due to lethal disruption of endosperm development was restricted to paternal excess, with maternal excess crosses yielding seeds with a germination frequency of 90% (Table 2). This was unexpected, since symmetrical triploid block is very widespread in angiosperms (Haig and Westoby, 1991). Whether a species expresses triploid block is related to that breeding system, with self-incompatible outbreeding species most likely to have triploid block, and self-compatible inbreeding species least likely (Brandvain and Haig, 2005). This weak inbreeder, strong outbreeder (WISO) pattern is consistent with the predictions of the parental conflict model for the evolution of genomic imprinting (Haig and Westoby, 1991). *Brassica* is, in general, an obligate outbreeder due to the operation of a strong sporophytic self-incompatibly system (Tantikanjana *et al.*, 2010). The expression of partial triploid block by the self-compatible isogenic 2*x*/4*x* accessions (BolSC2/4) used in the present work is therefore consistent with their recent derivation from a self-incompatible progenitor population (CrGC; Williams and Hill, 1986) and provided ideal material for examining the molecular mechanisms responsible for endosperm failure due to parental genome imbalance.

The behaviour of parental genomes in developing seeds resulting from imbalanced crosses may be influenced in two ways. The first is due to differential expression of maternal and paternal alleles depending on their parent-of-origin. The second is due to the differential dosage effect of the parental genome or ploidy (Bushell *et al.*, 2003). Attributing parent-of-origin effects to imbalanced parental ploidy alone therefore requires isogenic diploid and autotetraploid lines. The present study made use of genetically very well defined, homozygous near-isogenic and self-compatible diploid and tetraploid material in *Brassica* (BolSC2/4). In the study of Howard (1939), the material was derived from callus cuttings that contained a mixture of diploids and autotetraploids. Selfing the diploids produced a mixture of diploid and triploid individuals indicating a lack of genetic homogeneity. Håkansson (1956) omitted any description of his material, whereas Nishiyama and Inomata (1966) used two closely related subspecies of *B. rapa*, *chinensis* and *pekinensis*. We are therefore unable to assign their reported failure to obtain viable seed following imbalanced crosses to parental genome imbalance alone.

Our results agree in part with earlier reports of interploidy crosses in *Brassica*. We were successful in obtaining viable seeds from $4x \times 2x$ crosses at consistently twice the rate of that from a $4x \times 4x$ cross. Howard (1939) reported obtaining viable $4x \times 2x$ seeds whereas Håkansson (1956) and Nishiyama and Inomata (1966) did not. The latter studies also reported normal embryo development in early stages, which was generally interrupted, together with strong disturbance of endosperm development. In our study, the maternal excess crosses followed a similar pattern to that observed in Arabidopsis, with early cellularisation of the endosperm, and an inconspicuous chalazal endosperm (Scott *et al.*, 1998). Embryo development was advanced temporally, but final size was reduced compared to the embryos of self seed, consistent with reports in Arabidopsis (Scott *et al.*, 1998). However, triploid plants obtained from these seeds were sterile, which contrasts with results in Arabidopsis, where triploids are fertile (Henry *et al.*, 2005).

The paternal excess cross was not successful in *Brassica*, consistent with all previous reports in *Brassica* (Howard, 1939; Håkansson, 1956; Nishiyama and Inomata, 1966). We observed abnormal endosperm development and arrested globular/heart stage embryos in seeds resulting from a $2x \times 4x$ cross which do not appear to progress further (Figure 2j–l), as did Nishiyama and Inomata (1966) and Håkansson (1956). Håkansson (1956) described the endosperm in a $2x \times 4x$ seed as having a granular cytoplasm, which agrees with our observations of an abnormal endosperm with numerous vacuoles and a darkly staining cytoplasm by 19 DAP (Figure 2). Seeds obtained from mature siliques were shrivelled and concave, consistent with the absence of an embryo or cellularised endosperm.

The molecular mechanism underlying parent-of-origin effects in *Brassica*

A combination of mutant analysis and interspecific crossing experiments in Arabidopsis has provided most insights into the potential mechanism of parent-of-origin associated endosperm development effects, including triploid block (reviewed in Ishikawa and Kinoshita, 2009; Köhler *et al.*, 2010). However, Arabidopsis is an inbreeding, self-compatible species that, consistent with the WISO hypothesis, does not normally display triploid block (Scott *et al.*, 1998), suggesting that caution should be exercised in extrapolating any proposed mechanisms to other species, such as *Brassica*. Recently, however, more direct evidence has been provided by the Arabidopsis ecotype Columbia where, as described here for *Brassica*, triploid block is asymmetric, with lethality restricted to the $2x \times 4x$ cross (Dilkes *et al.*, 2008). The paternal excess endosperm displays massive endosperm over-proliferation and a failure of cellularisation, closely resembling that observed in $2x \times 6x$ crosses in other Arabidopsis ecotypes (Scott *et al.*, 1998) as well as in Arabidopsis × *Arabidopsis arenosa* crosses (Comai *et al.*, 2000; Bushell *et al.*, 2003). Interpretation of this phenotype together with earlier data has resulted in a potential general molecular model for triploid block based on regulation of pivotal events in endosperm development by a PcG complex (Ishikawa and Kinoshita, 2009; Köhler *et al.*, 2010). In the Arabidopsis endosperm the PcG complex represses (maternal) expression of *PHE1* and *AGL62* (Kang *et al.*, 2008). These genes are predicted to increase endosperm size by delaying cellularisation (Ishikawa and Kinoshita, 2009). The PcG complex can lose control over endosperm development as a consequence either of loss-of-function maternal mutations in its component genes, e.g. *mea* or *fis2*, or due to its 'titration' by additional target genes as occurs when an extra paternal genome is present in the endosperm following a $2x \times 4x$ cross. Consistent with this model, several members of the PcG complex (FIS2 and MEA) are imprinted, with expression restricted to the maternal allele (Köhler *et al.*, 2010). Further support is provided by Wolff *et al.* (2011), wherein many MADS genes, including those encoding AGL23, -28, -36, -96 and PHE1, that interact with AGL62 (Kang *et al.*, 2008), were expressed in a reciprocally imprinted and accession-dependent manner in the endosperm following crosses between Arabidopsis Col-0 and Bur-0 accessions.

Our analysis of *BolC.AGL62* expression in reciprocal $2x/4x$ interploidy crosses provides evidence to support the PcG titration model. In the balanced crosses, *BolC.AGL62* transcript was present at relatively high levels at 13 DAP, prior to the onset of endosperm cellularisation, and had declined approximately 10-fold by 19 DAP, at which point the endosperm was extensively cellularised (Figures 2a–f and 5). This pattern is consistent with that reported for wild-type diploid Arabidopsis (Kang *et al.*, 2008), where *AGL62* expression levels and cellularisation are negatively correlated. Maternal excess seed generated by the $4x \times 2x$ cross displayed precocious cellularisation (Figure 2g–i). Consistent with this, *BolC.AGL62* expression was already 10-fold lower than the control crosses at 13 DAP, and declined to near undetectable levels by 19 DAP (Figure 5a). Conceivably, the production of viable seed requires a certain level of *AGL62* expression to allow endosperm proliferation prior to early cellularisation. The negative correlation between *BolC.AGL62* transcript level and cellularisation was maintained in $2x \times 4x$ paternal excess seed, where *BolC.AGL62* expression was maintained at a relatively high level across all time points, and cellularisation failed to occur. This suggests that, as in Arabidopsis, the behaviour of the endosperm in interploidy crosses is in part regulated by the activity of *AGL62*, which in turn is controlled by a repressive PcG complex. Significantly, the phenotype of $4x \times 2x$ endosperms indicates that maternal excess provides additional PcG complex that suppresses *AGL62* expression to force precocious cellularisation and partly phenocopy an *agl62* mutation.

The behaviour of endosperm in paternal excess seed

Maternal excess crosses in both *Brassica* and Arabidopsis result in small, precociously cellularised endosperms that are phenotypically very similar (Figure 2; Scott *et al.*, 1998). However, although seed failure due to paternal excess in the two species is strongly associated with the absence of endosperm cellularisation, there are clear differences in other aspects of endosperm behaviour. In Arabidopsis, extreme paternal excess due to a $2x \times 6x$ cross, maternal *fis*/*msi* mutations or *MET1*antisense × 4*x*, produce seeds that display massive over-proliferation of chalazal endosperm cysts and transformation of peripheral endosperm into chalazal-like endosperm, which similarly over-proliferates (Scott *et al.*, 1998; Adams *et al.*, 2000; Köhler *et al.*, 2010). This behaviour has been attributed to the global deregulation of imprinted genes, such a *PHE1*, resulting from the loss of FIS PcG in imbalanced endosperms (Jullien and Berger, 2010; Köhler *et al.*, 2010). In contrast, in *Brassica* $2x \times 4x$ seeds the chalazal endosperm proliferates up to 60% but the peripheral endosperm does not undergo transformation or over-proliferation. One possible explanation is that over-expression of PHE1-like activity does not occur in *Brassica* as a result of paternal excess. Consistent with this is our failure to identify a *PHE1* homologue in any of the available *Brassica* EST collections or genome sequences (Table S1). Bemer *et al.* (2010) analysed the expression of 60 Type 1 MADS box genes in Arabidopsis and found that a majority are expressed in the female gametophyte or developing seed. In addition, several of these genes function in a redundant manner and act either independently or in heterodimers. The situation may be further complicated in diploid *Brassica* species, where three copies of a gene are often present due to sequential ancestral duplication events.

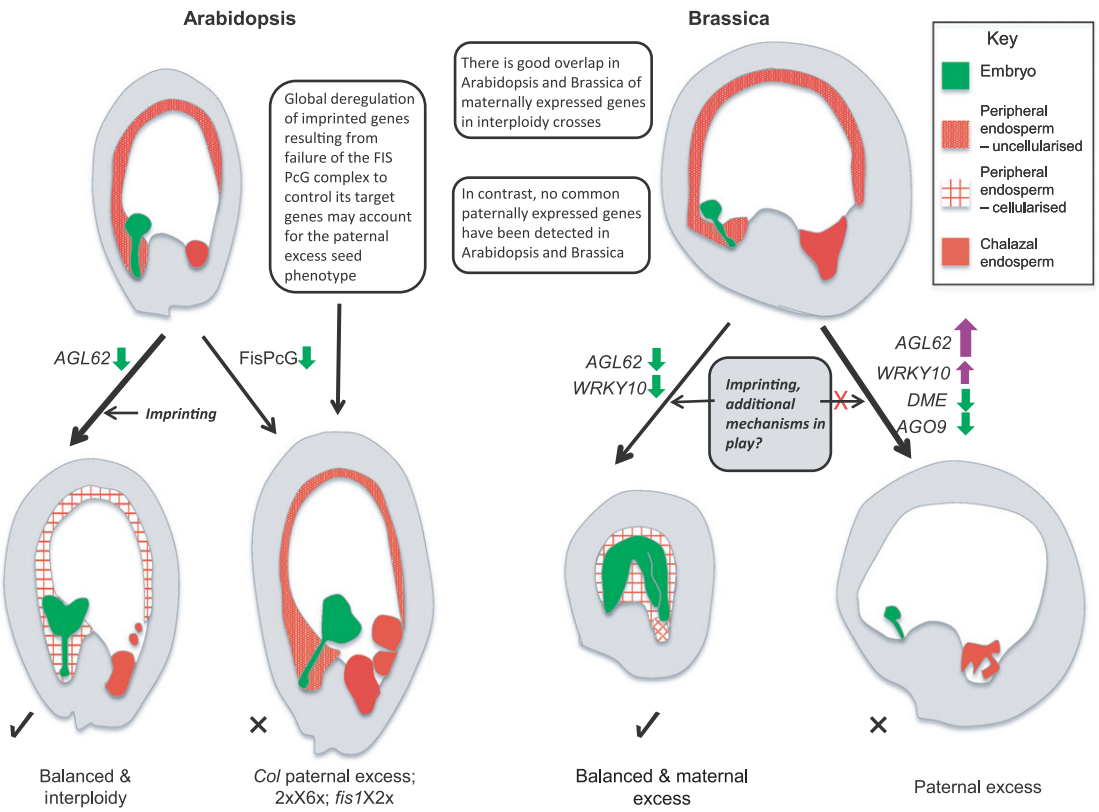

Figure 6. Molecular model for triploid block in Arabidopsis and *Brassica*.

Another significant difference was the increase of endosperm and seed size in crosses resulting from paternal excess in Arabidopsis in comparison with *Brassica*. In a preliminary analysis, we found no significant increase in transcript abundance of cyclin gene *BoIC.CYCD4:2*, which in Arabidopsis is associated with seed growth (Tiwari *et al.*, 2010). This lack of increase in cyclin transcript may be an explanation for the lack of over-proliferation observed in *Brassica*.

In Arabidopsis, the global deregulation of imprinted genes resulting from the failure of the FIS PcG complex to control its target genes (MEGs and PEGs) may account for the paternal excess seed phenotype (Jullien and Berger, 2010; Hsieh *et al.*, 2011; Wolff *et al.*, 2011). Since $2x \times 4x$ crosses in *B. oleracea* resulted in a paternal excess seed phenotype with features of extreme paternal excess in Arabidopsis, we compared the differentially expressed genes in *Brassica* paternal excess with the known MEGs and PEGs from Arabidopsis to determine whether a similar mechanism operates in *Brassica*. Consistent with this proposition was the down-regulation in $2x \times 4x$ endosperm of *Brassica* orthologues of the Arabidopsis MEGs *AGO9* and *AGL96*. Whilst the behaviour of MEGS in $2x \times 4x$ endosperm fitted the pattern established in Arabidopsis, we did not identify any PEGs that were up-regulated genes in our *Brassica* dataset. This further strengthens the idea that mechanisms in addition to down-regulation of FIS complexes play a role in determining the development of endosperm produced by interploidy crosses (Figure 6).

In conclusion, we found that *B. oleracea* displays asymmetric triploid block similar to the Columbia ecotype of Arabidopsis, despite the recent derivation of the *Brassica* lines from self-incompatible progenitors. The similarity of the behaviour of a naturally outbreeding species, *B. oleracea*, with Arabidopsis, Col-0, an inbreeder remains intriguing. *Brassica* demonstrates similar key pathways in the regulation of seed development as observed in Arabidopsis, wherein MADS-box genes play a significant role in controlling endosperm and hence seed development. There may have been some divergence in the family of MADS box genes affecting the precise nature of heterodimers formed in *Brassica* as compared with Arabidopsis. The down-regulation of MEGs in $2x \times 4x$ endosperm appears similar in both *Brassica* and Arabidopsis, but the behaviour of PEGs appears different in the two species.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The 2*x* and 4*x* *Brassica* lines were generated by anther culture (D. Pink, University of Warwick, Wellesbourne, UK) from an inbred line derived from a rapid-cycling population, CrGC121. The ploidy level was verified by flow cytometry (Plant Cytometry Services, Schijn-

del, the Netherlands). Plants were grown in 75% peat:12% loam:3% vermiculite:10% grit under controlled conditions at 18–15°C with 16 h light at 300 μmol m^{-2} sec^{-1}. Seedlings were transplanted in 9-cm pots, 14–17 days after initial potting. In order to limit the spread of pollen, inflorescences were enclosed in microperforated pollination bags prior to anthesis.

Pollinations

Semi-restricted pollinations were carried out on the main inflorescence. Buds were emasculated 1 day prior to anthesis. The individual pollinated flowers were tagged.

Phenotypic measurements

Seeds from individual siliques were placed on damp filter paper within 9-cm Petri dishes and incubated at 20°C. Germination was assessed by counting the number of seeds that had germinated (radicle emergence) 7 days after imbibition. Seed weight was measured using an analytical balance (Sartorius, BP211D, Epsom, Surrey, UK).

Ploidy analysis

Fresh leaf material 2–3 cm^2 was harvested and sent to Plant Cytometry Services for ploidy analyses.

Light microscopy

For sectioning, seeds were dissected as before and placed in fixative (4% paraformaldehyde in 0.1 M Sorenson's buffer, pH 7.0, 0.05% Tween-20). A gentle vacuum was applied to remove air bubbles. Samples were fixed overnight on a rotating shaker, washed three times in PBS buffer, followed by dehydration in an ethanol gradient and subsequently infiltrated using Technovit 7100 resin (TAAB Laboratories Equipment Ltd., Aldermaston, Berkshire, UK). Images of sections were captured using a Nikon camera (Nikon UK Ltd., Kingston Upon Thames, Surrey, UK), montages assembled using CorelDraw(Corel UK Ltd., Maidenhead, Berkshire, UK)/Photoshop (Adobe Systems Europe Ltd, Maidenhead, Berkshire, UK). Measurements were performed using ImageJ software (http://rsbweb.nih.gov/ij/). Images of mature seed were photographed using a Leica camera DFC300FX attached to a Leica MZ8 stereomicroscope (Leica Microsystems Ltd., Milton Keynes, Buckinghamshire, UK).

Seed size measurements

Seed size was determined using a Marvin digital seed analyser (Selecta Machinefabriek B.V., Enkhuizen, the Netherlands). Seed numbers and individual seed data were recorded using MARVIN 4.0 software (circularity setting of three).

Real-time quantitative PCR

Seed for three biological replicates was collected at the described time points and frozen in liquid nitrogen. The RNA was extracted using an RNeasy kit (Qiagen Ltd., Crawley, West Sussex, UK) and assessed for quality and quantity (Nanodrop® 1000; Thermo- Fisher Scientific, Loughborough, UK). The RNA was treated with TURBO DNA-*free* (Ambion, Life Technologies Ltd., Paisley, UK). Complementary DNA was prepared from 500 ng of RNA using SuperScriptIII enzyme (Invitrogen, Life Technologies Ltd., Paisley, UK) and primed with $Oligo(dT)_{20}$ (Invitrogen).

Relative transcript abundance was determined using the Platinum(R) SYBR(R) Green qPCR SuperMix-UDG kit (Invitrogen) on a 7500 RealTime PCR system (Applied Biosystems, Warrington, UK). Data were obtained from three biological and technical replicates for each sample. The C_t and reaction efficiency was determined using LINREGPCR v.11.1 (Ruijter *et al.*, 2009), relative expression values calculated using the Pfaffl equation (Pfaffl, 2001). Gene of interest expression was normalised against *BolC.GAPC* (*Bra016729*). Details of primers and gene models used are provided in Table S2.

Transcriptome analyses

Total RNA for transcriptome analysis was extracted from 16-DAP $2x \times 2x$ and $2x \times 4x$ seeds as above. Transcriptome sequencing was performed on the Illumina HiSeq 2000 platform (by Source BioScience, Nottingham, UK) to obtain 100-bp paired-end reads.

The RNA sequences obtained were aligned to *B. rapa* gene model, ensemble version 61 (http://brassicadb.org) using BOWTIE (Langmead *et al.*, 2009) and TOPHAT (Trapnell *et al.*, 2009) programs. Differential expression between the two transcriptomes was calculated using CUFFLINKS (Trapnell *et al.*, 2010) (See Table S4).

ACKNOWLEDGEMENTS

This work was supported by the UK Biotechnology and Biological Sciences Research Council (grant no. BB/F009721/1 and Rothamsted Institute Strategic. Programme Grant).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Magnified images of the chalazal endosperm following balanced and interploidy crosses.

Figure S2. Molecular phylogenetic analysis of PHERES and closely related MADS proteins from Arabidopsis and *Brassica rapa*.

Table S1. *Brassica* gene homology sequence matches for relevant *AGL* candidates.

Table S2. Table of primers.

Table S3. Comparison of reference genes.

Table S4. Bioinformatic analyses of transcript data.

Tables S5–S17. Transcript analyses of $2x \times 2x$ and $2x \times 4x$ seed.

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