Introduction

In mammals and plants, mothers differentiate distinctive structures specialized in the transport of maternal nutrients to the embryo, the mammalian placenta and the plant seed endosperm [1]. Thus, unilateral maternal contribution of nutrients results in an imbalanced parental contribution to the offspring. Such imbalance has been considered, in the frame of the kinship theory, as a potential cause for parental conflict of interest over allocation of resources to embryos [2,3]. This hypothesis has gained support in mammals and in plants from the effects of parental genome dosage imbalance on embryo growth in plants and animals [4–8]. These observations were followed by the discovery of imprinted genes, which are expressed by a single parental allele [1,9,10]. The overall effect of loss-of-function of imprinted genes conforms to the prediction of the parental conflict theory [27]. The parental conflict hypothesis was supported to a certain extent [2,9,10,12–16], computational analyses on the origin of the selection of imprinting at the MEA locus did not lead to unequivocal support [17–19]. However, the response to dosage imbalance is likely involved in deregulation of imprinted genes leading to sexual reproductive barriers [14] as suggested by studies involving Arabidopsis relatives [20,21]. Although recent evidence suggested that a mutation causing the production of diploid male gametes deregulates imprinted gene expression when crossed to diploid wild type [22], the expression of imprinted genes in response to genome dosage imbalance in a wild type Arabidopsis background remained to be tested in order to provide experimental evidence for the parental conflict theory in plants.

Currently the regulation of five maternally expressed imprinted genes have been characterized in Arabidopsis, the Polycomb Group (PcG) gene MEDEA (MEA) [23], the gene MATERNALLY EXPRESSED PAR C-TERMINAL (MPC) [24], the PcG gene FERTILIZATION INDEPENDENT SEED 2 (FIS2) [25], the transcription factor FWA [26], and the actin regulator FORMIN5 [27]. The overall effect of loss-of-function of MEA and FIS2 causes enhanced endosperm growth [28,29] leading to the conclusion that these two genes represent potential MIGs as predicted by the parental conflict hypothesis. By contrast, FORMIN5 loss of function leads to a reduction of endosperm growth and does not conform to the prediction of the parental conflict theory [27]. The transcription factor PHERES1 (PHE1) is a paternally expressed imprinted gene in Arabidopsis, which could play a role as a PEG
Parental Genome Dosage Deregulates Imprinting

Author Summary

In mammals and plants, imprinted genes are expressed preferentially by the copy inherited from either the mother or the father. In plants, genome dosage is easily manipulated using tetraploid plants that contain twice the genome dosage of the natural diploid plants. The increased maternal dosage reduces seed size while increased paternal dosage has the opposite effect. It was further proposed that parental genomic imbalances are directly mirrored by antagonistic regulations of imprinted genes encoding maternal growth inhibitors and paternal growth enhancers. However, these hypotheses were never tested directly. We measured the expression of imprinted genes and their regulators, in crosses between diploid and tetraploid Arabidopsis plants. Surprisingly, parental dosage imbalance affected each imprinted gene in a different manner and the imprinted status was also affected. Our results point to a relationship between imprinting and dosage imbalance that is more complex than predicted.

Additional imprinted genes have been characterized in Arabidopsis [32] but their function remains to be determined.

Plant reproduction is initiated by a double fertilization event [33]. Two haploid sperms are delivered to the female gametes, the egg cell and the central cell. Fertilization of the haploid egg cell leads to embryoogenesis. The second sperm cell fuses with the central cell producing the endosperm. The endosperm can be considered as an embryonic annex, which nurtures embryo development [10]. Parental imbalance of genome dosage in maize affects endosperm growth, which in turn influences embryo and seed growth [4, 8]. In Arabidopsis, increased maternal genome dosage in seeds resulting from crosses between ovules from tetraploid plants and pollen from diploid plants (4nmat × 2npat) leads to production of smaller endosperm, embryo and seed [5]. Reciprocal crosses (2nmat × 4npat) cause the opposite effect. These results have suggested that collectively increased dosage of the expressed maternal allele of MIGs reduces endosperm growth while increased dosage of the expressed paternal allele of PEGs increases endosperm growth [11].

Although it was assumed that parental dosage imbalances would be directly mirrored by variations in the expression of the PEGs and MEGs [2, 3, 11, 14], it became apparent that ME4 and PHE1 expression were regulated by FIS2 [31, 34–36]. This cross-regulation between imprinted genes could thus impact on the expression levels of ME4, FIS2, FWA, and PHE1 in seeds resulting from interплод crosses. We performed quantitative RT-PCR to assess the expression of imprinted genes in endosperm produced by crosses between diploid and tetraploid plants and observed a global deregulation of expression levels of imprinted genes accompanied by an unexpected loss of parental imprinting for somess genes. However, the expression of known key regulators of imprinting were not affected. Our results suggest that parental dosage imbalance disrupts imprinting through interactions between imprinted genes and other unidentified regulators.

Results/Discussion

Increased paternal dosage causes silencing of FIS2 controlled by DNA methylation

Increased paternal dosage is expected to increase the level of expression of the active maternal allele of FIS2 and FWA. Conversely, the global level of expression of these genes should not be affected by an increased dosage of inactive paternal alleles. We used quantitative RT-PCR to investigate the effect of increased parental dosages in crosses between tetraploid and diploid plants. We measured the expression at 2 days after pollination (2DAP) when the imprinted genes studied are highly expressed and control the timing of endosperm development [28]. Between fertilization and 2 days after pollination the developmental pattern and size of endosperm size is not affected, [5] suggesting that we could observe direct consequences of parental genome imbalances. We performed the experiments in two genetic backgrounds G24 (Figure 1, Table S1) and Columbia (Col) (Figure S1, Table S3) and obtained similar results. We observed variations of higher amplitude in Col background and conservatively took into account only significant changes observed in both backgrounds and supported by statistical tests (Tables S2 and S4). We investigated the effects of genome dosage imbalance in non-imprinted genes expressed in the seed as GaIPC or more specifically in endosperm as MIN3 [37] and did not observe significant fluctuation of their expression levels (Figure 1E and 1F and Figure S1E and S1F). Similarly, we did not observe significant changes in the expression of the two essential regulators of imprinting encoding DEMETER (DME) [38] and the DNA METHYLTRANSFERASE1 (MET1) [25, 26, 39] (Figure 1C and 1D and Figure S1C and S1D). The expression of these two genes is also not imprinted (data not shown). Our measurements thus indicated that parental genome dosage imbalance did not affect transcription globally and did not affect regulators of DNA methylation, which control imprinting.

We tested the effect of genome dosage imbalance on maternally expressed imprinted genes FIS2 and FWA, which are silenced by DNA methylation [25, 26]. We observed hardly any changes in levels of FWA expression (Figure 1B and Figure S1B). By contrast to FWA, FIS2 expression levels were very sensitive to parental genome imbalance. Levels of expression in self-fertilized 2n and 4n crosses were comparable (Figure 1A). As expected, maternal dosage of active maternal FIS2 alleles produced by (4nmat × 2npat) crosses increased FIS2 mRNA levels in endosperm (Figure 1A). Surprisingly, although (2nmat × 4npat) crosses did not change the dosage of transcriptionally active maternal FIS2 alleles, FIS2 expression was reduced in comparison to seeds produced by self-fertilized diploid plants. (Figure 1A). We obtained similar results in Columbia background (Figure S1A). A similar decrease of FIS2 expression was reported from 3 to 5 DAP in Landsberg erecta background using the meiotic jasmon (jas) mutant, which produces a proportion of diploid pollen [22]. Increased paternal dosage also reduced expression of the transcriptional reporter FIS2-GUS, which contains the transcriptional regulatory cis-elements required for imprinting regulation [25, 40] (Figure S2). Thus, increased paternal genome dosage down-regulates FIS2 expression irrespective of its genomic context.

Trans-silencing in Arabidopsis and maize [41–44] has been associated with the production of small interfering RNAs [45]. However, non-coding RNAs have not been shown to affect FIS2 expression and the down-regulation of FIS2 expression in response to increased dosage of inactive paternal alleles likely result from a distinct mechanism. We propose that the unexpected silencing of the paternal alleles of FIS2 in endosperm produced by (2nmat × 4npat) crosses could originate from increased paternal dosage of a paternally expressed imprinted inhibitor of FIS2 or from the activity of yet unidentified cis-elements.

Interplloid crosses cause deregulation of imprinting

We assessed the imprinted status of FIS2 and FWA in interplloid crosses. Both genes remained strictly maternally expressed in (4nmat × 2npat) crosses (Figure 2A and 2B). This indicated that the
increased dosage of active maternal alleles was directly responsible for the increased expression levels of FIS2. The opposite (2n_{mat} x 4n_{pat}) crosses did not affect the FWA imprinting status (Figure 2B) but caused an unexpected paternal expression of FIS2, resulting in the loss of FIS2 imprinting (Figure 2A). Loss of FIS2 imprinting was not restricted to RLD 2n X Col 4n crosses as it also occurred in crosses using Ler 2n and C24 2n (data not shown). We thus concluded that increased paternal dosage decreases the overall expression of FIS2 while both parental alleles become expressed. Such rather paradoxical effect is difficult to interpret. A negative interaction between MET1 activity, which maintains silencing marks and the trans-silencing mechanisms activated by the increased dosage of silenced paternal allele may cause removal of the silencing marks on the paternal allele of FIS2. Alternatively in response to reduced FIS2 expression, a transcriptional activator of FIS2 might be over-expressed and overcome silencing of the paternal allele.

We tested whether parental genome dosage imbalance would also deregulate imprinting of the genes MEA and PHE1. MEA was predominantly expressed from the maternal allele in (4n_{mat} x 2n_{pat}) crosses as in control diploid crosses (Figure 2C). Surprisingly in (2n_{mat} x 4n_{pat}) crosses the expression from the maternal allele decreased causing a predominant paternal expression of MEA leading to an apparent inversion of MEA1 imprinting expression (Figure 2C). PHE1 imprinted status was not altered in response to paternal genome increase (Figure 2D). However PHE1 imprinting is hardly observed in crosses between Col females and C24 males [31], and we could not assess the effect of increased maternal dosage on PHE1 imprint (Figure 2D). In conclusion we observed that at least two out of four genes studied lost imprinting as a result of dosage imbalance. These results suggest that increasing the dosage of the silenced allele of an imprinted gene causes the removal of the imprinting marks on the silenced allele.

**Dosage imbalances effect on MEA and PHE1 indicate crosstalk between imprinted gene regulations**

We further tested the effect of dosage imbalance on the maternally expressed imprinted gene MEA, which is silenced by PcG mediated H3K27 trimethylation of its paternal allele [35,36]. Although MEA is maternally expressed, MEA expression was repressed when the maternal genome dosage increased in (4n_{mat} x 2n_{pat}) crosses in C24 background (Figure 3A, Table S1

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Figure 1. Effects of interploid crosses on the expression of DNA methylation-dependent imprinted genes. Quantitative PCR measurements of FIS2 (A), FWA (B), DME (C), MET1 (D), GAPC (E), and MINI3 (F) mRNAs were performed on total mRNAs extracted from siliques produced by crosses between diploid and tetraploid parents (2 DAP, C24 ecotype). Each point represents the average RQ value obtained for four independent biological samples (values can be found in Table S1). Error bars represent the standard deviation. * represents p < 0.05 of Student’s t-test using C24 2X2 as a reference. All p values can be found in Table S2. The genome copy number in the endosperm is represented inside each bar (active copy in white, inactive copy in grey).

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and Table S2) but not in Col background (Figure S3A, Table S3 and Table S4). A modest increased MEA expression in response to increased paternal dosage was observed in Col background (Figure S3A). A mild increase was also observed at 1 DAP in Ler background using jas mutant mimicking (2n_mat×4n_pat) crosses [22].

Parental dosage imbalances strongly perturbed PHE1 expression following the trends exhibited by MEA expression levels (Figure 3B and Figure S3B) although PHE1 is paternally expressed. A strong increase of PHE1 expression was also observed after 3 DAP in Ler background using jas mutant as pollen donor [22]. These results could be explained by the common regulation of MEA and PHE1 by the PcG complex, which contains FIS2 and MEA and is active in endosperm [35,36,46]. FIS2 encodes a Suppressor of zeste 12 (Su(z)12) Polycomb group subunit [47]. Since the two other members of the Su(z)12 family are not expressed in Arabidopsis endosperm [40] the reduction of FIS2 expression levels in (2n_mat×4n_pat) crosses could become limiting for Polycomb group activity, leading to increased expression of MEA and PHE1. This effect would also be directly responsible for the inversion of MEA imprinting (Figure 3C) as previously shown for the effect of reduced Polycomb activity in loss of function mutants for FERTILIZATION INDEPENDENT ENDOSPERM (FIE) [35,36].

We verified that parental dosage imbalance and tetraploidy do not affect FIE expression levels (Figure 3C and Figure S3C). We further tested whether alterations of FIS2 expression would mimic the effects observed on MEA and PHE1 in response to parental genome dosage imbalance. We used the loss of function allele fis2-6 to decrease the levels of FIS2 expression and a transgenic line expressing a complementing FIS2-YFP fusion protein to increase the levels of FIS2 expression [48] (Figure 4, Table S5).

Manipulating FIS2 mRNA levels (Figure 4A) did not affect FWA expression (Figure 4B). We did not observe any effect of increased FIS2 levels on PHE1 expression levels (Figure 4C). However we observed that decreased FIS2 expression causes increased PHE1 expression but did not affect MEA expression. Despite the fact that MEA and PHE1 are over-expressed in response to decreased FIS PcG activity [36], [46].

We conclude that increased FIS2 expression caused by maternal genome dosage increase is not directly responsible for the decreased expression of MEA and PHE1. By contrast, paternal genome dosage causes an unexpected decrease of FIS2 expression, which in turn could directly or indirectly increase PHE1 expression. As an alternative explanation, increased dosage of PHE1 copy number might rather directly increase PHE1 expression in response to paternal dosage increase.

Figure 2. Effect of interploid crosses on imprinted status. (A) The imprinting of FIS2 is detected by a size polymorphism between the strains RLD and Columbia (Col). The parent indicated at the top is the mother. (B) The imprinting of FWA is detected by a restriction polymorphism between the strains Ler and Col. (C) The imprinting of MEA is detected by a restriction polymorphism between the strains RLD and Col. (D) The imprinting of PHE1 is detected by a restriction polymorphism between the strains C24 and Col. GAPDH is used as control. The band quantification is represented below each gel as a percentage of the GAPDH band intensity.
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Figure 3. Effects of interploid crosses on the expression of genes imprinted by Polycomb group activity. Quantitative PCR measurements of MEA (A), PHE1 (B), and FIE (C) mRNAs were performed on total mRNAs extracted from siliques produced by crosses between diploid and tetraploid parents (2 DAP, C24 ecotype). Each point represents the average RQ value obtained for four independent biological samples (values can be found in Table S1). Error bars represent the standard deviation. * represents p<0.05 of Student’s t-test using C24 2X2 as a reference, p values can be found in Table S2. The genome copy number in the endosperm is represented inside each bar (active copy in white, inactive copy in grey).

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Conclusions

Reciprocal changes of parental dosage do not cause the symmetrical variations of expression of imprinted genes predicted by previous studies. A similar complex phenomenon was observed in mammals [48–52]. However parthenogenetic embryos used in mice to investigate parental dosage imbalance do not allow a direct test for the interactions between the paternal and maternal allele. In addition parthenogenotes are produced via complex in vitro manipulations and other factors may perturb silencing at imprinted loci. In plants dosage imbalances are created in vivo in undisturbed reproductive tissues and their consequences are unlikely to reflect the consequence of experimental manipulations. In response to parental imbalance we observed unexpected non-symmetrical deregulation of the expression of imprinted genes coupled with a loss of imprinting in two out of four imprinted genes studied. The modulation of seed size by dosage imbalance does not result in variations of PEGs and MIGs expression parallel to variation of the dosage of the respective parental genome. In addition, the mode of perturbation may vary during later development stages in endosperms produced by crosses involving jas mutant that produces a fraction of diploid pollen [22].

After 6 DAP, paternal excess dosage causes endosperm developmental defects similar to loss of FIS complex activity [5]. The fact that MEA ectopic expression rescues late endosperm developmental defects in crosses with jas pollen [22] suggests that the late perturbations of imprinted genes expression in response to jas pollination may result rather from an indirect deregulation of endosperm developmental timing caused by loss of FIS activity [28]. At early stages of endosperm development we do not observe a strong link between FIS2 expression and the perturbation of MEA and PHE1 expression. Thus our data do not support that the FIS PcG complex directly deregulates imprinted genes expression in response to dosage imbalance a couple of days after fertilization. In addition parental genome dosage imbalance does not affect expression of FIE, the essential component of the FIS PcG complex. Parental genome dosage imbalance does not affect expression of the regulators of DNA methylation MET1 and DME. Hence, parental dosage imbalance does not directly affect the known major controls of imprinting. Nevertheless, we propose that parental dosage imbalance deregulates FIS2 and MEA expression, which causes late endosperm developmental defects including over-proliferation and ectopic expression of PHE1. A similar phenotype has been observed in crosses between A. thaliana and A. arenosa. Such deregulation compromise seed viability and likely contribute to species isolation mechanisms involving tetraploidization [21], [43], [53].

We do not currently understand the mechanisms that cause the immediate response to dosage imbalance and deregulation of FIS2, MEA and PHE1 expression. Such mechanisms could involve other controls of DNA methylation [54] or small non-coding RNAs inherited maternally [55] or paternally [56]. The apparent parental conflict linked to imprinting in plants and in mammals likely results from a complex series of non-symmetrical regulations during zygotic development. Nevertheless these mechanisms could involve imprinted regulators controlled in a dosage dependent manner predicted by the kinship theory [2], [57].

Materials and Methods

Plant lines

The wild-type control lines C24, Col, Ler, and RLD were obtained from the ABRC stock center. The tetraploid lines in C24 and in Col ecotypes were kindly provided by Rod Scott [5] and Luca Comai [58]. The reporter line pFIS2-GUS (C24 accession) was kindly provided by Abed Chaudhury [40]. FIS2YFP line was kindly provided by Ramtin Yadegari [48] and fs2-6 was previously identified in our laboratory [59].

Allele-specific RT–PCR and quantitative real-time RT–PCR

Siliques two days after pollination (2DAP) were collected from Arabidopsis plants and frozen in liquid nitrogen. Total RNAs were extracted using the RNeasy mini kit (Qiagen). After DNase
treatment using DNase free kit (Ambion), RNAs were reverse-transcribed using Stratascript RT kit (Stratagene).

Allele-specific RT-PCR reactions were performed as previously described [23,25,26,60]. Band quantification was performed using the ImageJ software (http://rsbweb.nih.gov/ij/).

Real-time PCR assays were performed using a PCR Master Mix (Applied Biosystems, Foster City, CA). One µl of RT product was used to perform each PCR reaction. Amplification reaction was carried out using specific primers at a concentration of 0.5 mM in a 10 µl reaction. Sequence of specific primer pairs can be found in Table S6. The specificity of the amplification product was determined by performing a dissociation curve analysis. PCR efficiency was determined using the LinReg program [61]. The PCR reaction and quantitative measurements were achieved with 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling parameters were 2 min at 50°C, 10 min at 95°C and 50 cycles of 15 sec at 95°C, 60 sec at 60°C. We performed four biological replicates, with three technical replicates for each sample. For each PCR reaction the ΔCt was calculated using ACT11 gene as endogenous control except for Figure 4 where FIS2 was used as endogenous control. Relative Quantitation values (RQ) were calculated using the 2^−ΔΔCt method [62]. Values given in Figure 1, Figure 3, Figure 4, Figure S1 and Figure S3 represent the average of RQ values obtained for four or three biological replicates for each point and the error bars represent the standard deviation.

Supporting Information

Figure S1  Effects of interploid crosses on the expression of DNA methylation dependent imprinted genes (A-D). Quantitative PCR measurements of FIS2 (A), FWA (B), PHE1 (C), and MEA (D). mRNAs were performed on total mRNAs extracted from siliques produced by crosses between C24 and fis2-6 parents (2 DAP, C24 ecotype) to test for the effect of FIS2 down-regulation and between Col and FIS2YFP (2 DAP, Col ecotype) to test the effect of increased FIS2 expression. Each point represents the average RQ value obtained for three independent biological samples. Error bars represent the standard deviation. * represents p<0.05 of Student’s t-test using Col 2X2 as a reference, # represents p<0.05 of Student’s t-test using Col 2X2 as a reference, p values can be found in Table S4.

Figure S2  Effects of interploid crosses on the expression FIS2 transgenes. Effect of an increased maternal dosage on expression of transcriptional reporter pFIS2-GUS expression at 1.5 DAP. Staining was stopped before signal saturation and three classes of
seeds were distinguished on the basis of the intensity of signal. The percentage of each class in crosses between ovules of the marker line and wild-type pollen from diploid or tetraploid plants is indicated below each corresponding micrograph. Scale bars correspond to 25 μm.

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**Figure S3** Effects of interploid crosses on the expression of genes imprinted by Polycomb group activity. Quantitative PCR measurements of MEA (A), PHE1 (B), and FIE (C) mRNAs were performed on total mRNAs extracted from siliques produced by crosses between diploid and tetraploid parents (2 DAP, Col ecotype). Each point represents the average RQ value obtained for four independent biological samples (Table S3). Error bars represent the standard deviation. * represents p<0.05 of t-test using Col2X2 as a reference, p values can be found in Table S4. Found at: doi:10.1371/journal.pgen.1000885.s003 (0.75 MB TIF)

**Table S1** RQ value of the C24 experiment after normalisation with Act11. C24 2nX2n sample 1 was normalised to 1. Each RQ value in this table corresponds to the average RQ value of 3 technical replicates. Found at: doi:10.1371/journal.pgen.1000885.s004 (1.32 MB TIF)

**Table S2** Probability values obtained after a student’s t-test on C24 sets of crosses from Figure 1 and Figure 3. Two samples are significantly different when p<0.05. Found at: doi:10.1371/journal.pgen.1000885.s004 (1.32 MB TIF)

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**Table S3** RQ value of the Columbia experiment after normalisation with Act11. Col 2nX2n sample 1 was normalised to 1. Each RQ value in this table corresponds to the average RQ value of 3 technical replicates. Found at: doi:10.1371/journal.pgen.1000885.s006 (1.34 MB TIF)

**Table S4** Probability values obtained after a student’s t-test on Col sets of crosses from Figure S1 and Figure S3. Two samples are significantly different when p<0.05. Found at: doi:10.1371/journal.pgen.1000885.s007 (1.31 MB TIF)

**Table S5** Probability values obtained after a student’s t-test on qPCR results shown in figure 4. Two samples are significantly different when p<0.05. Found at: doi:10.1371/journal.pgen.1000885.s008 (0.76 MB TIF)

**Table S6** List of primers used in this study. Found at: doi:10.1371/journal.pgen.1000885.s009 (1.12 MB TIF)

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**Author Contributions**

Conceived and designed the experiments: PEJ FB. Performed the experiments: PEJ. Analyzed the data: PEJ. Wrote the paper: FB.
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