Imprinting of the Polycomb Group Gene MEDEA Serves as a Ploidy Sensor in Arabidopsis

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Abstract

Balanced maternal and paternal genome contributions are a requirement for successful seed development. Unbalanced contributions often cause seed abortion, a phenomenon that has been termed “triploid block.” Misregulation of imprinted regulatory genes has been proposed to be the underlying cause for abnormalities in growth and structure of the endosperm in seeds with deviating parental contributions. We identified a mutant forming unreduced pollen that enabled us to investigate direct effects of unbalanced parental genome contributions on seed development and to reveal the underlying molecular mechanism of dosage sensitivity. We provide evidence that parent-of-origin–specific expression of the Polycomb group (PcG) gene MEDEA is causally responsible for seed developmental aberrations in Arabidopsis seeds with increased paternal genome contributions. We propose that imprinted expression of PcG genes is an evolutionary conserved mechanism to balance parental genome contributions in embryo nourishing tissues.

Introduction

Polyplody, the presence of more than two complete sets of chromosomes within an organism, is known to be common in plants and in some animals such as amphibians, fish and reptiles [1,2]. The widespread occurrence of polyploids among plant species suggests that polyploidy is evolutionary beneficial and represents a major mechanism for plant adaptation and speciation [2–6]. The additional sets of chromosomes may originate from the same species (“autopolyploid”), or from the hybridization of two different species (“allopolyploid”). Polyploids can arise spontaneously by the fusion of diploid gametes with a normal haploid gamete, resulting in the formation of a triploid zygote. Diploid gamete formation resulting from failure of reduction during meiosis occurs in several plant species and can give rise to triploids that serve as a bridge to the formation of stable polyploids with an even set of chromosomes [4].

In most flowering plants the fusion of one sperm cell with the haploid egg cell is accompanied by the fusion of a second sperm cell with the homodiploid central cell nucleus, resulting in the formation of the triploid endosperm with a 2:1 ratio of maternal to paternal genomes. The endosperm is a nourishing tissue supporting embryo growth [7]. Double fertilization occurs also in polyploids, resulting in the formation of embryo and endosperm with proportionally increased ploidies. However, crosses between plants of different ploidy often fail because seed development does not proceed normally and non-viable seeds are produced, a phenomenon that has been termed “triploid block” [8]. It is assumed that abnormalities in growth and structure of the endosperm are the cause of triploid seed failure [9], consistent with the proposed role of the endosperm in reproductive isolation and angiosperm speciation [10–12]. In many species the 2:1 ratio of maternal to paternal genomes in the endosperm is required for normal seed development [12,13], giving rise to the hypothesis that gene dosage effects and imprinting of regulatory genes in the endosperm is the underlying cause for developmental failure in seeds with deviating parental contributions [9,14,15]. Genomic imprinting is the mitotically stable inheritance of differential expression states of maternal and paternal alleles caused by different epigenetic modifications of the alleles. Genomic imprinting renders maternal and paternal genomes non-equivalent, and balanced contributions of maternal and paternal genomes are therefore essential for post-fertilization development [15–17].

The penetrance of the interploidy hybridization barrier varies within and between species. Whereas maize is particularly sensitive to changes in the ratio of maternal to paternal genome contributions in the endosperm [13,14], in Arabidopsis thaliana a substantial accession-dependent variation in the degree of postzygotic seed lethality has been observed in crosses between individuals of different ploidy [18,19]. The phenotype of viable seeds resulting from reciprocal interploidy crosses is different, depending on which parent contributes the higher genome dose. Whereas seeds developing from a cross of a diploid female with a tetraploid male develop larger endosperm, the reverse is true when the maternal plant contributes the double genome dose [19]. Increased endosperm growth in seeds with increased paternal genome contribution is associated with an increased number of mitotic...
Results

The jason Mutant Forms Enlarged Triploid Seeds with Increased PHE1 Expression

PHERES1 (PHE1) is a direct target gene of the Arabidopsis thaliana FIS PcG complex and is predominantly expressed in the endosperm during early stages of seed development [31]. PHE1 expression ceases around the time of endosperm cellularization, when the embryo has reached late heart stage. We performed a genetic screen aimed at identifying novel regulators of PHE1 expression and made use of a previously established reporter line containing the PHE1 promoter fused to the β-GLUCURONIDASE (GUS) reporter [31]. The identified jason (jas-1) mutant had strongly increased GUS staining in the endosperm starting at 5 to 6 days after pollination (DAP), when embryos had reached late heart stage, whereas the GUS staining pattern was indistinguishable from the wild type during early seed development (Figure 1A–1H). Increased GUS staining was restricted to the endosperm, apparent staining of the embryo is a consequence of the endosperm overlaying the embryo. We tested whether increased expression levels of the reporter gene were reflected by increased expression of the endogenous PHE1 gene and analyzed PHE1 expression at defined DAP (Figure 1I). Consistent with the results from the PHE1 reporter construct, increased endogenous PHE1 expression was observed from 5 DAP onwards.

PHE1 is an imprinted gene that is predominantly paternally expressed [27]. Therefore, we investigated whether increased expression of PHE1 in jas mutant seeds is caused by breakdown of PHE1 imprinting. We crossed the Arabidopsis accession C24 as female with pollen from Landsberg erecta (Ler) wild type or jas mutant and analyzed allele-specific expression of PHE1 in seeds resulting from these crosses. However, no increase in maternally derived PHE1 transcripts was detected in jas mutant seeds and increased PHE1 transcript levels were solely established by increased expression of the paternal PHE1 allele (Figure 1K).

Development of jas seeds is delayed compared to wild-type seeds: whereas wild-type seeds reached maturity at 10 DAP, jas seeds reached maturity only after about 18 DAP (Figure 1G and 1H). Mature jas seeds were significantly increased in size (Figure 2A and 2B), resembling seeds derived from 2n×4n crosses [19]. Therefore, we tested whether the jas mutation caused triploid seed formation by measuring the ploidy levels of the progeny of diploid homoyzogous jas plants. Indeed, among the jas progeny we found diploid and triploid (45%) but no tetraploid seedlings (10 triploids among 22 plants; Figure 2C). Seedlings grown from size-selected enlarged jas seeds were all triploid, indicating that enlarged seeds are indeed triploid (n = 51). These data suggest that the jas mutation causes diploid gamete formation at high frequency, however, the presence of diploid seedlings as well as normal sized seeds among the progeny of jas plants (Table 1) indicates that the jas mutation is not completely penetrant.

The jas mutation is sporophytic recessive; mutant plants were detected at a ratio of about 25% among segregating F2 plants (n = 185; $\chi^2 = 0.134 < \chi^2_{0.05|1} = 3.84$), indicating normal transmission of the jas allele through male and female gametes. Also, JAS does not appear to have a general role in seed development, as abnormal seed development was not observed in seeds from selfed jas heterozygous plants, even though 25% of the seeds are homoyzogous for the jas allele. In order to test whether the jas mutation affects male or female gametogenesis, we reciprocally crossed jas plants with Ler wild-type plants and analyzed seeds developing from these crosses. Enlarged seed formation was only observed when the jas mutation was paternally transmitted (Table 1 and Figure S1), suggesting that jas pollen is diploid. Conversely, we did not detect significant numbers of abnormally-sized seeds when the jas mutation was maternally transmitted (Figure S1), strongly suggesting that jas only affects male gametogenesis. Additional support for this conclusion was derived from the finding that all F1 seedlings derived from crosses of jas plants with wild-type pollen were diploid (n = 40). Together, triploid seed formation in the
identified jas mutant is caused by a defect during male gametogenesis leading to unreduced gamete formation.

The jason Mutant Forms Unreduced Male Gametes at High Frequency

Diploid pollen is significantly larger than haploid pollen [32], and consistent with our hypothesis that jas pollen is diploid, we observed 62% enlarged pollen in jas plants (n = 144; Figure 3A and 3B). Furthermore we measured DNA content of sperm cells from wild-type and enlarged jas pollen. The mean fluorescence from the enlarged pollen was approximately twice that of the wild-type and of normal-sized jas pollen, indicating that the former has twice the DNA content and is thus diploid (Figure 3C). To define the stage of pollen formation affected by the jas mutation, we analyzed the meiotic products of jas microspore mother cells. Whereas wild-type plants almost exclusively formed tetrads, jas plants formed dyads and triads at high frequency (64% dyads, 19% triads, n = 307 Figure 3D–3F). Thus, JAS is required in the sporophyte to regulate male meiosis and a meiotic defect is responsible for the formation of diploid pollen in jas. Consistent with this view we frequently observed ten chromosomes in dyad microspores, in contrast to the five chromosomes observed in tetrad microspores (Figure 3G and data not shown). The subsequent mitotic divisions are not affected by the jas mutation; all pollen grains formed by jas mutant plants contained two sperm cells and one vegetative cell (Figure 3B).

Figure 1. Overexpression of PHE1::GUS and the endogenous paternal PHE1 allele in seeds of jas plants. (A,C,G) PHE1::GUS expression in wild-type seeds. (B,D,F,H) PHE1::GUS expression in seeds of selfed homozygous jas plants. Seeds were analyzed at 4 (A,B), 5 (C,D), 6 (E,F) and 10 DAP (G,H). Scale bars, 100 μm. (I) Quantitative RT–PCR analysis of PHE1 expression in wild-type and jas seeds. Error bars, s.e.m. (K) PHE1 imprinting is not affected in jas seeds. Allele specific PHE1 transcript levels were determined after crosses of C24 with wild-type (Ler) or jas plants [27]. DAP: days after pollination. wt: wild type.

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To define the meiotic stage affected by the jas mutation, we analyzed chromosome behavior during male meiosis in jas mutants. Chromosome spreads showed that meiosis in the jas mutant progressed normally and was indistinguishable from wild-type meiosis until the end of telophase I. Synapsis was complete and chiasmata as well as bivalents formed (Figure S2A, S2B, S2C, S2G, S2H, S2I). At metaphase II, however, we observed differences to wild-type meiosis. Whereas wild-type chromosomes aligned into two well separated metaphase II plates (Figure S2D), jas chromosomes failed to align properly (Figure S2J), likely

Figure 2. Homozygous jas plants produce significantly larger triploid seeds. (A) Silique from wild-type and jas plants after self-fertilization. Triploid jas seeds are enlarged (white asterisks) and sometimes abort (dark brown seeds). Lower panels show ripe seeds. Scale bars, 0.5 mm upper panels, 1 mm lower panels. (B) Seed weight of wild-type and jas plants. Average seed weight is given in mg per 50 seeds. Significance was determined by two-tailed Student’s t-test, *P < 0.01. Numbers above bars indicate numbers of scored seeds per genotype. Error bars, s.e.m. (C) Representative flow cytometry histogram plots of nuclei from wild-type, tetraploid (4n) and seedlings grown from enlarged jas seeds. doi:10.1371/journal.pgen.1000663.g002
causing a failure in chromatin separation at the second meiotic division (Figure S2K) and the formation of dyads (Figure S2L) and triads (Figure S2M), in contrast to predominantly occurring tetrad formation in wild-type (Figure S2N).

We map-based cloned the JAS gene and found the jas mutation to cause a premature stop codon in the fifth exon of the At1g06660 gene, encoding an as yet unknown protein without domains of described functions (Figure 4A and 4B). BLAST searches of Arabidopsis proteins revealed a single related protein (At2g30820; JAS-LIKE) with 64% similarity. JAS was predominantly expressed during reproductive development, whereas JAS-LIKE has a broader expression domain (Figure 4C), suggesting partially redundant functions of both genes. JAS homologs were not identified in animals; however, JAS is conserved throughout the plant kingdom and contains a highly conserved domain at the C-terminus (Figure 4D). We identified three independent T-DNA lines containing insertions in intron 1 (jas-2), exon 5 (jas-3) and exon 3 (jas-4). Mutant alleles jas-3 and jas-4 caused dyad and triad formation at comparable frequency as the jas-1 mutant (Figure 3E), confirming that the identified mutation is indeed the cause of the jas phenotype. The frequency of dyad formation in the jas-2 allele was approximately half that of the other alleles (data not shown), indicating that the jas-2 mutant allele is not completely penetrant. We also generated and analyzed F1 jas-1/jas-3 plants. While plants heterozygous for either the jas-1 or jas-3 allele did not produce dyads, we clearly observed dyad formation in F1 plants containing both alleles (Figure 3E). The presence of dyads in multiple jas alleles and in plants containing both the jas-1 and jas-3 alleles confirms that mutations in the JAS gene are indeed the cause of the jas phenotype.

### Table 1. Seed phenotype of reciprocal crosses between jas and wild-type plants.

<table>
<thead>
<tr>
<th>Female</th>
<th>Ler (selfed)</th>
<th>jas (selfed)</th>
<th>jas</th>
<th>Ler</th>
</tr>
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<tbody>
<tr>
<td>Male</td>
<td>×</td>
<td>×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>9%</td>
<td>11%</td>
<td>4%</td>
<td>10%</td>
</tr>
<tr>
<td>Normal</td>
<td>91%</td>
<td>51%</td>
<td>95%</td>
<td>42%</td>
</tr>
<tr>
<td>Enlarged</td>
<td>0%</td>
<td>36%</td>
<td>0%</td>
<td>45%</td>
</tr>
<tr>
<td>Aborted</td>
<td>0%</td>
<td>2%</td>
<td>1%</td>
<td>3%</td>
</tr>
<tr>
<td>n (seeds)</td>
<td>406</td>
<td>758</td>
<td>918</td>
<td>737</td>
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Green or dry siliques resulting from self pollination or out-crosses to wild-type Ler plants were opened and the seeds classified as normal, enlarged, aborted, or unfertilized ovules. n (seeds), number of seeds scored.
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Attributed to the fact that only about half of these seeds are triploid (Table 1), reducing the statistical power to detect small expression changes, whereas all seeds derived from tetraploid pollen parents are triploid. Strikingly, 53% of up-regulated genes and 41% of down-regulated genes in seeds derived from jas pollen parents were also up- or down-regulated in mutants lacking FIS function (p = 7.04E-106 for up-regulated genes and p = 7.56E-49 for down-regulated genes; Figure 5A). Similarly, 89% of up-regulated and 54% of down-regulated genes in fis2 mutants were as well up- or down-regulated in triploid seeds derived from tetraploid pollen parents (p = 9.38E-164 for up-regulated genes and p = 2.86E-57 for down-regulated genes; Figure 5A). There was a strong linear relation with a slope close to 1 when comparing fold changes of jas or fis2 seeds to fold changes of seeds derived from tetraploid pollen parents (Figure S3, slope parameter = 0.87 and 0.88, respectively). This demonstrates that not only was the set of differentially expressed genes very similar between the analyzed samples, but that also the magnitude of change was very similar in seeds with increased paternal genome contribution and in seeds lacking FIS PcG function. These findings strongly suggest that increased paternal genome contribution causes global deregulation of direct or indirect target genes of the FIS PcG complex. To substantiate these findings, we tested whether genes with altered expression in triploid seeds were enriched for H3K27me3 [31]. Indeed, genes that were up-regulated in triploid seeds or in seeds lacking FIS PcG function were significantly enriched for H3K27me3, supporting the idea that direct FIS PcG target genes are deregulated in triploid seeds (Figure 5B).

Interestingly, genes commonly up-regulated in the three datasets had a preferential expression in the endosperm and were found in all three endosperm domains (micropylar, peripheral and chalazal domains; Figure 5C). These findings suggest that increased paternal genome contribution as well as lack of FIS PcG function predominantly affects endosperm development. Furthermore, these data indicate that FIS PcG function is required to suppress genes in the endosperm that are required during the earlier stages of endosperm development. In contrast, genes that were commonly down-regulated in all three datasets were expressed in various plant organs, suggesting that developmental perturbations in triploid and fis2 mutant seeds are caused by reduced expression of genes that have a general role during plant development. This idea is supported by the observation that down-regulated genes in triploid seeds are significantly enriched for genes with functions in cellularization and cell cycle control (Table S2), two processes that have a general role during plant development.

Among the genes commonly up-regulated in all three datasets we found PHE1 as well as MEIDOS (MEO), a gene that we previously identified among genes with deregulated expression in fis mutants [31]. Furthermore, we identified the potential FIS target gene AGL62 [33] as well as several other AGL genes (AGL40, AGL36, AGL90; Table S1) with as yet unknown function which, however, were previously shown to interact with PHE1 (AGL40, AGL62) or with interaction partners of PHE1 (AGL36, AGL90) in yeast two-hybrid studies [36]. We tested MEO and AGL62 expression in jas seeds at 5–8 DAP and found strongly increased MEO and AGL62 expression levels (Figure 6A and 6B), supporting the results obtained from transcriptome profiling studies. Taken together, these findings strongly support the idea that increased paternal genome contribution causes de-repression of FIS target genes during late seed development.

### Triploid Seeds Have Decreased MEA mRNA Levels

Although the FIS subunit MEO is expressed until late stages of seed development in embryo and endosperm [28], a role for the FIS...
complex has only been established to date for female gametophyte and early seed development [37,38]. Our results suggest that the FIS complex is also required for gene repression in the endosperm during late seed development and that increasing the paternal genome contribution interferes with FIS function. One possible explanation for this finding is that imprinted components of the FIS complex that are only expressed maternally become limited in the endosperm containing an increased paternal genome contribution. We tested this hypothesis by analyzing expression of the paternally imprinted gene MEA [28,30]. We confirmed expression of MEA in wild-type seeds 5–8 DAP and, importantly, observed decreased expression levels of MEA in seeds of self-fertilized jas plants starting at 5–6 DAP (Figure 6C). Because transcript levels were normalized to ACTIN11 with bi-allelic expression, the increased paternal genome contribution in jas is expected to cause an apparent reduction in transcript levels for genes with only maternal expression; thus, the measured changes in relative MEA transcript levels were in the expected range. Reduced MEA transcript levels were not observed at earlier seed developmental stages, most likely because activation of the paternally contributed genome occurs with a delay of 3–4 days [39] and therefore, increased paternal genome contributions are unlikely to impact on the transcript level of maternally expressed genes.

Figure 3. Homozygous jas plants form dyads and enlarged diploid pollen. (A) Pollen from jas plants was mixed with wild-type pollen marked by the sperm cell marker MGH3-H2B-GFP [65]. GFP negative enlarged pollen grain is derived from jas plants. Scale bar, 10 μm. (B) DAPI staining of pollen shown in panel (A). Enlarged jas pollen contains two sperm nuclei (sn) and one vegetative nucleus (vn). (C) DNA content of sperm cells in mature pollen from wild type and jas plants. Bars show mean relative DNA contents (DAPI fluorescence values) for pollen from wild type (WT) or jas plants, with jas pollen classified on the basis of size; normal sized (jas) or enlarged (jas enlarged). The mean fluorescence from the enlarged pollen was approximately twice that of the wild type and normal sized jas pollen, indicating that it has twice the DNA content. wt; wild type. (D) Tetrad formation in wild-type plants. Scale bar, 25 μm. (E) Dyad formation in jas plants. Scale bar, 25 μm. Scale bar, 10 μm. (F) Quantification of meiotic products in Ler wild-type, jas-1/+, jas-1/−, jas-3/+, jas-3/−, jas-1/jas-3 and jas-4/−. Numbers on top of columns indicate numbers of analyzed meiotic products. Triads observed in wild type are most likely caused by spore superposition. (G) Chromosomal spreads of wild-type tetrad (left panel) and jas dyad (right panel). Chromosomes are marked by arrow heads.

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MEA is biallelically expressed in the embryo, but exclusively maternally expressed in the endosperm [28]. As embryo development in triploid jas seeds is delayed, we wondered whether decreased transcript levels of MEA in jas seeds were caused by reduced MEA expression in the embryo. Therefore, we tested allele-specific MEA transcript levels in seeds derived from crosses of accession RLD with wild-type Ler or jas pollen. We detected strongly decreased transcript levels of the maternal MEA allele and only
marginally decreased transcript levels of the embryo-derived paternal MEA allele in jas seeds (Figure 6D). Thus, the observed decreased MEA transcript levels in the endosperm were caused by reduced maternal-specific MEA transcripts.

FIS2 encodes a subunit of the FIS complex and is also regulated by genomic imprinting with only the maternal FIS2 allele being expressed in the endosperm [29,40]. We also analyzed expression of the FIS2 gene that like MEA had a second expression peak during late seed development (Figure 6E). However, in contrast to reduced MEA transcript levels in jas seeds, relative FIS2 transcript levels were increased (Figure 6E). We wondered whether increased FIS2 expression is caused by activation of the paternal
FIS2 allele. Therefore, we tested allele-specific FIS2 transcript levels in seeds derived from crosses of accession C24 with wild-type Ler or jas pollen. However, FIS2 remained imprinted in wild-type and jas seeds (Figure 6F), suggesting different regulatory modes of MEA and FIS2 regulation in triploid seeds. Together, our data suggest that developmental changes observed in triploid seeds are caused by lack of sufficient MEA rather than FIS2 expression.
Increased MEA Expression Normalizes Triploid Seed Development

If decreased MEA transcript levels are causally responsible for developmental aberrations in triploid seeds, increased MEA expression levels should normalize development of triploid seeds and partially suppress the jas phenotype. To test this hypothesis, we overexpressed MEA under control of the RPS5a promoter, which is expressed in embryo and endosperm [41]. Complementation of the mea/+ mutant with a hemizygous RPS5a::MEA transgene is expected to result in a reduction of the seed abortion ratio to 25%, as half of the mea mutant gametophytes will inherit the transgene. Expression of MEA under control of the RPS5a promoter in the mea/+ mutant (mea/+;RPS5a::MEA/+) suppressed the mea seed abortion phenotype, with 6 out of 14 lines showing seed abortion ratios between 29% (χ² = 2.34 < χ²₀.₀₁[1] = 6.64) and 31% (χ² = 6.52 < χ²₀.₀₁[1] = 6.64), which is close to the expected seed abortion ratio. This suggests that the RPS5a promoter is active at the correct stage and at sufficient strength and should be suitable to compensate reduced MEA transcript levels in most triploid seeds. We selected three transgenic lines being hemizygous for single locus insertions of the RPS5a::MEA construct in the jas mutant background that had either wild-type-like or strongly increased MEA expression levels (Figure 7A) and assayed the number of enlarged seeds. Consistent with our hypothesis, all lines had significantly less enlarged seeds (Figure 7B, Table S3, Figure S4), with two of the lines having less than half the number of enlarged seeds compared to the jas mutant.

We hypothesized that normalization of seed size by RPS5a::MEA expression would be associated with a progression of triploid embryo development. We tested this hypothesis by comparing developmental stages of jas and jas;RPS5a::MEA seeds. Gynoecia of jas and jas;RPS5a::MEA transgenic lines were manually pollinated and seed development inspected after 10 DAP. Indeed, all three RPS5a::MEA containing jas lines had reduced numbers of developmentally delayed seeds compared to the jas mutant not
containing the transgene (Figure 7C), indicating that increased MEA expression can normalize seed development in triploid jas seeds. In jas mutant plants, only segregating wild-type seeds had reached maturity at 10 DAP, whereas triploid jas seeds were either in the torpedo or walking stick stage (Figure 7D). We were not able to phenotypically differentiate mature triploid seeds containing the RP53a::MEA transgene from diploid transgene containing seeds (Figure 7D), suggesting that triploid seeds with increased MEA expression can complete seed development at similar pace as diploid seeds.

Finally, we asked whether increased MEA expression would normalize PHE1 expression in triploid seeds. Again, consistent with our hypothesis, PHE1 expression levels were significantly reduced by increasing MEA expression in the jas mutant (Figure 7E). Together we conclude that reduced MEA transcript levels are causally responsible for seed developmental aberrations in triploid jas seeds.

**Discussion**

Misbalanced expression of imprinted genes has long been implicated as the cause of seed development defects after interploidy crosses [9,14,15]. Our study provides strong evidence in favor of this hypothesis and demonstrates that MEA imprinting is a major origin of developmental defects caused by increased paternal genome contributions. In this work we took advantage of the jas mutant that forms unreduced diploid pollen at high frequency, which allowed us to create first generation polyploids and to investigate direct effects of chromosome doubling on seed development. Lack of JAS function causes failure of chromatin segregation during meiosis II, leading to second division nuclear restitution. This mechanism is different to the formation of unreduced pollen by parallel spindles during meiosis II or omission of the second meiotic division, as it occurs in the recently identified Atps1 and sand mutants, respectively [42,43]. It also clearly differs from the male defect of the switch1 (dyad) mutant that is defective in prophase I, leading to aberrant segregation of chromatids during the first meiotic division [44]. Thus, the identified JAS gene provides molecular insights into a novel mechanism of unreduced pollen formation in plants and will further our understanding of the underlying molecular mechanisms of polyploidy formation.

Similarities of the endosperm phenotype in triploid jas seeds and seeds lacking components of the FIS PcG complex let us to propose that FIS function is impaired in seeds with increased paternal genome contribution. We show that FIS genes MEA and FIS2 are expressed at later stages of seed development, concomitantly with the time of endosperm cellularization [45]. Endosperm cellularization in triploid Arabidopsis seeds with paternal excess is delayed or fails completely [18,19], consistent with increased expression of FIS target genes like PHE1 and its proposed interaction partner AGL62 [36], which is implicated to suppress endosperm cellularization [35]. This suggests that the FIS PcG complex has a function during later stages of seed development to suppress inhibitors of endosperm cellularization. We did not observe deregulation of FIS PcG target genes during early development of triploid jas seeds, indicating that only this later function of the FIS PcG complex is impaired by increased paternal genome dose in the endosperm. This suggest that the number of accessible FIS target sites increases at the time of endosperm fertilization, consistent with our finding that the paternal PHE1 allele becomes targeted and silenced by the FIS PcG complex at this time.

MEA and FIS2 are regulated by genomic imprinting and are only maternally expressed [24,28,30]. Therefore, we hypothesized that transcript levels of both genes could become limiting in triploid seeds with increased paternal genome contribution. The paternal MEA allele is silenced by the MEA containing FIS complex [46-48]. Therefore, reduced MEA transcript levels in tetraploid jas endosperm could potentially cause a breakdown of MEI imprinting, leading to a reactivation of the paternal MEA allele. However, we show that MEA remains imprinted in tetraploid endosperm, suggesting that MEA is able to recruit sufficient FIS complexes leading to stable silencing throughout seed development, while many other FIS target genes including MEO, AGL62 and the paternal PHE1 allele, are not. This implicates that different FIS target genes have different binding affinities for the FIS PcG complex, which is consistent with observations made in Drosophila that dependent on the genomic context, PcG proteins have different binding affinities to their targets [49]. At the PHE1 locus, different binding affinities of the FIS PcG complex to maternal and paternal alleles might be caused by a differentially methylated region located downstream of the PHE1 locus that is required for repression of maternal PHE1 alleles [50].

Although FIS2 remained imprinted in triploid jas seeds, FIS2 transcript levels were increased compared to wild-type seeds. This suggests that activation of maternal FIS2 alleles requires transcriptional activators that are induced by increased paternal genome dose in the endosperm. Support for this idea comes from a recent study of Jullien and colleagues [51], who propose the requirement of additional activating factors for FIS2 expression in the endosperm, based on the finding that lack of DNA methylation does not lead to FIS2 activation in vegetative tissues.

Increased MEA expression normalized triploid seed development and caused triploid embryo development to progress at similar pace like wild-type embryos. About half the number of triploid jas seeds were affected by increased MEA expression, indicating that either MEA expression levels were not sufficiently enhanced in all seeds, or, alternatively, that there are additional factors required to restore normal seed development. Expression of MEA under control of its endogenous promoter [25] as well as under control of the RP53a promoter (this study) significantly suppressed abortion of mea mutant seeds; however, there was a deviation from the expected seed abortion ratio of 4 to 7%, indicating that indeed transgene-derived MEA expression levels are not in all seeds sufficient to restore wild-type seed development.

Interestingly, postzygotic lethality of hybrids between A. thaliana and A. arenosa seems to depend on reduced expression of the FIS2 and increased expression of AGL family members AGL62 and AGL90, indicating that disturbed FIS complex function might contribute to hybrid seed failure as well [52]. Indeed, increased maternal genome dose strongly suppressed hybrid incompatibility in crosses of tetraploid A. thaliana and A. arenosa [53], suggesting that increased transcript levels of maternal-specific FIS genes permit normal seed development. Consistently, several other studies reported that there are reciprocal differences in interploidy crossing success, with unreduced eggs being more effective in polyploid formation than unreduced pollen [4], suggesting that increased dosage of PcG complexes is less detrimental for endosperm development than lack of sufficient PcG function. Whether indeed increased maternal genome dose causes repression of FIS PcG target genes will be subject of future investigation.

Maize endosperm is highly dosage sensitive and deviations from the 2:1 maternal to paternal genome dose will ultimately cause seed abortion [13,14]. The ME1 homolog Mez1 is also imprinted in the maize endosperm [54], suggesting that dosage sensitivity in the endosperm is caused by a conserved mechanism involving
imprinted expression of PcG genes. Finally, the Sfmbt2 PcG gene has recently been shown to be imprinted in trophoblast tissues of mouse embryos [55]. Trophoblast tissues are particularly sensitive to perturbations in genomic imprinting, reflected by dysmorphic trophoblast development in interspecies hybrids [56] and uniparental embryos [57]. Thus, it is possible that imprinting of PcG genes in embryo-nourishing tissues of flowering plants and mammals is an evolutionarily conserved system ensuring correct parental genome contributions in the developing progeny.

Materials and Methods

Plant Material and Growth Conditions

Plants were grown in a growth room at 70% humidity and daily cycles of 16 h light at 21°C and 8 h darkness at 18°C. The jas-1 allele was induced in the Landsberg erecta (Lei) accession by ethyl methanesulfonate mutagenesis and harbors a premature stop codon at amino acid position 294 (G-to-T nucleotide substitution at position +1960 of the genomic sequence). Unless otherwise stated, all experiments were performed with the jas-1 allele. Additional alleles in the Columbia (Col) accession were found in T-DNA insertion libraries: jas-2 (SALK_083575), jas-3 (SAIL_813_H03) and jas-4 (SALK_042866) harboring insertions in intron 1, exon 5, and exon 3, respectively. The jas-2 mutant (Lei accession) has been previously described [33]. Tetraploid lines of Ler were obtained from the Nottingham Arabidopsis Stock Centre. The RPS5a::ME4 overexpressing lines were generated by Agrobacterium tumefaciens-mediated transformation into jas-1 heterozygous plants and five transgenic lines homozygous for the jas-1 mutation were analyzed. The PHE1::GUS line has been described previously [31]. This line was used as the corresponding wild-type control for allele-specific expression analysis. For crosses, designated female partners were emasculated, and the pistils hand-pollinated one day after emasculation. For RNA expression analysis, three siliques were harvested at each of the indicated time points.

Genetic Screen and Mapping

The PHE1::GUS line, mutagenized by ethyl methanesulfonate (EMS) treatment, was screened for mutants by selecting M2 plants that showed GUS activity during late stages of seed development. For genetic mapping of the jas mutation, we established an F2 mapping population by crossing jas with the Col-0 accession. Analysing 280 jas F2 plants using PCR-based polymorphisms, the mutation was located on chromosome 1 in an area of 570 kb between polymorphisms SM104_106.6 and PAT1.2 on BACs T21E18 and F24B9, respectively. Open reading frames within this region were PCR-amplified and analyzed using the SURVEYOR Mutation Detection Kit (Transgenomic). A polymorphism was detected in At1g06560 (JAS) and confirmed by sequencing.

GUS Expression Analysis and Phenotypic Characterization of Seeds and Pollen

Siliques were harvested for GUS staining at the indicated time points. Staining of seeds to detect GUS activity was done as described previously [31]. Mature pollen nuclei were visualized after staining with 4,6-diamidino-2-phenylindole (DAPI) as described previously [58]. Buds were harvested for microscopic analysis of tetrad formation and fixed overnight in 3:1 ethanol:sodium citrate, pH 4.68 and incubated with 1% cytohelicase, 1% pectolyase and 1% cellulase in citric buffer for 3–4 hours at 37°C. Squashes were analyzed by a Zeiss Axioscope fluorescence microscope (Zeiss, Germany) equipped with a cooled CCD camera (Vistron, Germany). Images were acquired using MetaView software (Universal Imaging Corporation, USA).

Flow Cytometry

The ploidy levels of leaf cell nuclei were determined by flow cytometry using a FACSD flow analyser (Partec). Leaves were chopped with a razor blade in CyStain extraction buffer (Partec), filtered through a 30-µm CellTrics filter (Partec) into a sample tube, and stained with CyStain Staining buffer (Partec). Data were collected for approximately 10,000 nuclei per run and were presented on a linear scale.

Plasmid Constructs

The 1.6 kb upstream sequence of the RPS5a translational start was cloned into pB7WG2 vector replacing the 35S promoter. The ME1 cDNA was cloned into pENTR/D-TOPO (Invitrogen). The RPS5a::ME4 overexpressing construct was generated by cloning reaction (Invitrogen) between pB7WG2/Rps5a and pENTR/D-TOPO/ME4

RNA Extraction and qPCR Analysis

RNA extraction and generation of cDNAs were performed using RNAeasy Plant Mini Kit (Qiagen) according to the supplier’s instructions. Quantitative PCR was done on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems) according to the supplier’s recommendations. Quantitative RT-PCR was performed using three replicates and results were analyzed as described [59] using ACTIN11 as a reference gene. Briefly, mean expression values and standard errors for the reference gene as well as for the target genes were determined, taking into consideration the primer efficiency that was determined for each primer pair used. Relative expression values were determined by calculating the ratio of target gene expression and reference gene expression and error bars were derived by error propagation calculation. The primers used in this study are specified in Table S4.

Allele-Specific Expression Analysis

Primers for PHE1, ME1 and FIS2 allele specific expression analysis are specified in Table S2. Allele-specific FIS2 expression analysis was done by crossing C24 and Lei accessions. The amplified products were digested with AflIII and analyzed on a 2.5% agarose gel. Allele-specific ME1 and PHE1 expression was analyzed as described previously [27,28].

Microarray Analysis

Samples, array design, and hybridizations. The three largest flower buds of the inflorescences were emasculated, and the
gynoecia were pollinated one day later. Seeds of at least 20 siliques per sample and three independent biological replicates were harvested into 40 μl RNAlater (Sigma, Buchs, Switzerland) at 6DAP. Glass beads (1.25–1.55 mm) were added, and the samples were ground unrefined in a Silamat S3 (Ivoclar Vivadent, Elhvangen, Germany). RNA was extracted using the RNAqueous kit (Ambion/Applied Biosystems, Lincoln, CA) combined with Plant RNA Isolation Aid (Ambion/Applied Biosystems) according to manufacturer’s instructions. The RNA was subjected to a DNase treatment. cDNA was prepared from total RNA using the WT Ovation Pico System (NuGEN Technologies, San Carlos, CA) according to manufacturer’s instructions. SPIA amplification was used to prepare single-stranded cDNA from the mRNA starting material. Fragmented and biotin-labeled single-stranded cDNA targets were generated with the FL-Ovation cDNA Biotin Module V2 (NuGEN). Affymetrix Arabidopsis ATH1 GeneChips were used throughout the experiment (Affymetrix, Santa Clara, CA). The exact list of probes present on the arrays can be obtained from the manufacturer’s website (http://www.affymetrix.com). Analysis was based upon annotations compiled by TAIR (www.arabidopsis.org, version 2007-5-2). Data were deposited into the ArrayExpress database (Accession number E-MEXP-2316).

Bioinformatic analysis. All data processing was performed using the statistic package R (version 2.8.1) that is freely available at http://www.r-project.org/ [60]. Signal values were derived from Affymetrix CEL files using GCRMA. Quality control was done using the affyQCReport package in R. In addition, we calculated coefficients of variation (cv) between replicates as a quantitative measure of data quality and consistency between replicates as described previously [31]. Median cv values for triplicate array sets were between 1.4 and 1.8% demonstrating the high quality of the data. Because in jas and fis2 only about 50% of the seeds are mutant, observed fold changes (FCobs) were smaller than real FC (FCreal) in mutant seeds. FCreal values were estimated using equation FCreal = 2 × FCobs − 1.

Differentially expressed genes were identified using the limma package in R [61]. Multiple-testing correction was done using the q-value method [62]. Probe sets were called significantly differentially expressed when q < 0.05. To correct for the increased statistical power of the 2n×4n cross, where not only 50% but all seeds are affected, q = 0.075. To compensate for the 2n×4n cross, where not only 50% but all seeds are affected, q = 0.05 was required in this case. To enrich for biologically relevant changes, only probe sets with FCreal > 2 or FCreal < −2 were selected. Differentially expressed genes were grouped into functional gene ontology categories (obtained from www.arabidopsis.org). Data for H3K27m3 target loci were from [34]. The significance of enrichment was estimated based on the hypergeometric test and multiple-testing correction according to [63] with a critical p-value of 0.01. Analysis of tissue-specificity of differentially expressed genes was performed in Genevestigator [64].

Supporting Information

Figure S1 Paternal transmission of the jas mutation causes changes in seed size. (A) Seed size histogram of seeds derived from self-fertilized wild-type plants and self-fertilized jas mutants. (B) Seed size histogram of seeds derived from self-fertilized wild-type plants and jas mutants fertilized with wild-type pollen. Mean size of corresponding wild-type seeds was set to one. For each sample 151 seeds were analyzed.

Table S1 Deregulated genes in triploid jas seeds and seeds derived from crosses of 2n×4n plants. Found at: doi:10.1371/journal.pgen.1000663.s004 (0.54 MB PDF)

Table S2 Functional characterization of genes deregulated in seeds at 6DAP derived from crosses of wild-type plants with pollen from either jas plants or tetraploid plants as well as manually self-pollinated fis2 mutants.

Table S3 Seed phenotypes of wild-type, jas, and transgenic lines jas;RPS5a::MEA+. Green or dry siliques were opened and the seeds classified as normal, enlarged, aborted, or unfertilized ovules. n (seeds), number of seeds scored. P values were determined by Chi-square test comparing normal and enlarged seeds of jas and transgenic lines. N.a.; not applicable.

Table S4 Primers used in this study.

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Author Contributions

Conceived and designed the experiments: AE CK. Performed the experiments: AE LB VE MR LH. Analyzed the data: AE LB VE MR OMS LH CK. Contributed reagents/materials/analysis tools: DT OMS. Wrote the paper: AE LB LH CK.

References


