Endosperm cellularization defines an important developmental transition for embryo development

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SUMMARY
The endosperm is a terminal seed tissue that is destined to support embryo development. In most angiosperms, the endosperm develops initially as a syncytium to facilitate rapid seed growth. The transition from the syncytial to the cellularized state occurs at a defined time point during seed development. Manipulating the timing of endosperm cellularization through interploidy crosses negatively impacts on embryo growth, suggesting that endosperm cellularization is a critical step during seed development. In this study, we show that failure of endosperm cellularization in fertilization independent seed 2 (fis2) and endosperm defective 1 (ede1) Arabidopsis mutants correlates with impaired embryo development. Restoration of endosperm cellularization in fis2 seeds by reducing expression of the MADS-box gene AGAMOUS-LIKE 62 (AGL62) promotes embryo development, strongly supporting an essential role of endosperm cellularization for viable seed formation. Endosperm cellularization failure in fis2 seeds correlates with increased hexose levels, suggesting that arrest of embryo development is a consequence of failed nutrient translocation to the developing embryo. Finally, we demonstrate that AGL62 is a direct target gene of FIS Polycomb group repressive complex 2 (PRC2), establishing the molecular basis for FIS PRC2-mediated endosperm cellularization.

KEY WORDS: Arabidopsis, Endosperm, Polycomb group proteins

INTRODUCTION
Seed development in flowering plants is initiated by double fertilization of the female gametophyte. The female gametophyte harbors two distinct gametic cells that will have distinct fates after fertilization: the haploid egg cell will give rise to the diploid embryo and the homodiploid central cell will form the triploid endosperm (Drews and Yadegari, 2002). The endosperm is a terminal tissue that supports embryo growth by delivering nutrients acquired from the mother plant (Ingram, 2010). Like most angiosperms, the endosperm of Arabidopsis thaliana follows the nuclear type of development, in which an initial phase of free nuclear divisions without cytokinesis (syncytial phase) is followed by cellularization (Costa et al., 2004). In Arabidopsis, at the 16-nuclei stage the syncytium begins to differentiate into three distinct developmental domains: the micropylar, central and chalazal domains. The presence of a large central vacuole forces the cytoplasm in the central domain into a thin peripheral layer, while syncytial cytoplasm surrounds the embryo in the micropylar domain and the chalazal endosperm develops adjacent to the vascular connection with the seed parent (Berger, 2003; Brown et al., 1999; Brown et al., 2003). At the eighth mitotic cycle, cellularization of the syncytial endosperm is initiated in the micropylar domain around the embryo, coinciding with the early heart stage of embryo development (Boisnard-Lorig et al., 2001). Cellularization proceeds in a wave-like manner from the micropylar to the chalazal domain, filling the central cell, except for near the cyst in the chalazal chamber, with successive layers of endosperm cells (Brown et al., 1999; Berger, 2003; Brown et al., 2003).

Compartmentalization of the developing seed into three distinct endosperm domains is likely to regulate the uptake of nutrients into the developing seed. Experiments with labeled sucrose in oilseed rape seeds suggest that the transport of sucrose from the integuments to the embryo occurs via the micropylar endosperm, while sucrose uptake via the chalazal endosperm is thought to be involved in filling the central endosperm vacuole, the main storage pool for hexoses during seed development (Morley-Smith et al., 2008). The large central vacuole determines sink strength during early development by rapidly converting imported sucrose to hexoses in the young seed. With progressing endosperm cellularization, the size of the central vacuole decreases, correlating with a decrease in the ratio of hexoses to sucrose (Ohto et al., 2005; Morley-Smith et al., 2008; Ohto et al., 2009).

Endosperm cellularization is impaired in several mutants affecting cytokinesis in the embryo, including knolle, hinkel, open house, runkel and pleiade (Sorensen et al., 2002), implying that endosperm cellularization and somatic cytokinesis share multiple components of the same basic machinery. The spätzle mutant is characterized by the absence of cellularization in the endosperm but does not show cytokinesis defects in the embryo, indicating a role for SPÄTZE in a process specific to endosperm cellularization (Sorensen et al., 2002). Similarly, in the endosperm defective 1 (ede1) mutant, the endosperm fails to cellularize but the effects on embryo patterning are less severe, implicating a main function for EDE1 in endosperm cellularization (Pignocchi et al., 2009). However, in both mutants, embryo development is delayed after the heart stage and seed shrinkage or collapse occurs, suggesting that endosperm cellularization is crucial for normal embryo and seed development (Sorensen et al., 2002; Pignocchi et al., 2009). Interploidy crosses furthermore support an important role for endosperm cellularization in embryo development.

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Increased paternal genome dosage results in the delay or complete failure of endosperm cellularization, correlating with impaired embryo development (Scott et al., 1998; Dilkes et al., 2008; Erilova et al., 2009). Developmental defects caused by interlopy crossovers are associated with a dysregulation of genes that are directly or indirectly controlled by the Fertilization independent seed (FIS) Polycomb group (PcG) complex, implicating that developmental aberrations in response to interlopy crossovers are largely caused by dysregulated FIS target genes (Erilova et al., 2009).

PcG proteins form chromatin-modifying complexes that ensure the long-term controlled repression of specific target genes. PcG proteins form several families of multiprotein complexes, including the two main complexes Polycomb repressive complex 1 (PRC1) and PRC2. PRC2-mediated gene repression is characterized by trimethylation of lysine 27 of histone H3 (H3K27me3) and seems to primarily control genes involved in developmental decisions (Hennig and Derkacheva, 2009; Beisel and Paro, 2011). The subunits of PRC2 complexes are evolutionary well conserved. In plants, several PRC2 subunits are encoded by small gene families that form specific complexes with distinct functions during plant development (Hennig and Derkacheva, 2009). The FIS PRC2 complex plays a pivotal role in suppressing the initiation of endosperm and seed development in the absence of fertilization (Ohad et al., 1996; Chaudhury et al., 1997; Köhler et al., 2003a; Guitton et al., 2004). After fertilization, loss of FIS function causes endosperm overproliferation and cellularization failure, ultimately leading to seed abortion (Chaudhury et al., 1997; Kiyosue et al., 1999; Sorensen et al., 2001). A major regulator of endosperm cellularization is the type I MADS-box transcription factor AGAMOUS-LIKE 62 (AGL62) (Kang et al., 2008). Loss of AGL62 causes precocious endosperm cellularization, indicating a role for AGL62 as a suppressor of endosperm cellularization. Whereas in wild-type seeds AGL62 expression declines abruptly prior to cellularization, in fis mutants AGL62 expression does not decline (Kang et al., 2008), suggesting that FIS PRC2 might regulate endosperm cellularization by controlling the expression of AGL62.

In this study we investigated the role of FIS PRC2 in endosperm cellularization and the consequences of endosperm cellularization failure. We show that failure of endosperm cellularization in fertilization independent seed 2 (fis2) and ede1 mutants correlates with impaired embryo development. Restoration of endosperm cellularization in fis2 seeds by reducing AGL62 expression promotes embryo development, strongly supporting an essential role of endosperm cellularization in viable seed formation. We furthermore reveal that endosperm cellularization failure in fis2 seeds correlates with increased hexose levels, suggesting that arrest of embryonic development is a consequence of failed nutrient translocation to the developing embryo. Finally, we establish a molecular link for FIS PRC2-mediated endosperm cellularization by showing that AGL62 is a direct target gene of the FIS PRC2 complex.

MATERIALS AND METHODS

Plant material and growth conditions

The fis2 mutant alleles used in this study are fis2-1 (Ler accession) (Chaudhury et al., 1997) and fis2-5 (Col-0 accession) (Weinroth et al., 2010). The ede1-1, agl62-2 and osd1-1 mutant alleles are in the Col-0 accession background and have been described previously (Kang et al., 2008; Pignocchi et al., 2009; d'Erfurth et al., 2009). For crosses, designated female partners were emasculated, and the pistils hand-pollinated 2 days after emasculation. Single- and double-mutant plants were characterized by PCR using the primers listed in supplementary material Table S1. Seeds were surface sterilized (5% sodium hypochlorite, 0.01% Triton X-100) and plated on MS medium (MS salts, 1% sucrose, pH 5.6, 0.8% Bactoagar). After stratification for 1 day at 4°C, plants were grown in a growth room under a long-day photoperiod (16 hours light and 8 hours darkness) at 23°C. Ten-day-old seedlings were transferred to soil and plants were grown in a growth room at 60% humidity and daily cycles of 16 hours light at 23°C and 8 hours darkness at 18°C. The APL3::GUS line was generated by Agrobacterium tumefaciens-mediated transformation (Clough and Bent, 1998) into heterozygous fis2-1 plants and six independent lines homozygous for the transgenic locus in the fis2-1 background were analyzed.

Transmission analysis

Seeds were surface sterilized as above and plated on MS media supplemented with 100 μM gibberellic acid to facilitate germination. After stratification at 4°C in the dark for 3 days, seedlings were grown in a growth room under a long-day photoperiod (16 hours light and 8 hours darkness) at 23°C. Seedlings were harvested for genotyping after 12-16 days using the primers listed in supplementary material Table S1.

Embryo rescue and in vitro culturing

Silicques of fis2 mutant plants were collected at ~8-10 DAP and surface sterilized for 10 minutes in 1.5% sodium hypochlorite and 0.1% Tween 20 and then washed three times with sterile water. Embryos of fis2 seeds were dissected under sterile conditions using insulin needles. The embryos were plated on Murashige and Skoog medium (MS salts, pH 5.8, 0.8% Bactoagar, 2.7 mM glutamine) containing either 30 mM or 340 mM sucrose and cultivated under long-day conditions at 21°C.

Genomic DNA extraction from embryos was performed using the REDExtract-N-Amp Plant PCR Kit (Sigma, Poole, UK) with the primers listed in supplementary material Table S1.

Generation of plasmids

The 1.8 kb sequence upstream of the APL3 (AT4G39210) transcriptional start was amplified by PCR using the primers listed in supplementary material Table S1 and cloned into pCAMBIA1381Z (Cambia).

Analysis of carbohydrates

For each genotype and time point, seeds of three silicques were collected, corresponding to three biological replicates. Only undamaged seeds were harvested on ice and counted during harvesting, allowing relation of the measured amounts of carbohydrates to the number of seeds. The seeds were ground in a Silamat S5 mixer (Ilovcar Vivadent, Anherst, USA) twice for 10 seconds and extracted with 200 μl warmed 80% ethanol for 4 minutes at 80°C under constant rotation. After centrifugation for 5 minutes at 16,000 g, the supernatant was transferred to a new tube and the extraction repeated. The combined supernatants were dried under vacuum at 30°C.

The dried samples were resuspended in 50 μl water and soluble sugars were measured in a MWGt Discovery XS-R microplate reader (BioTek Instruments, Winooski, USA) after adding 50 μl of cocktail [25 mM HEPES pH 7.5, 1 mM MgCl2, 1 mM ATP, 1 mM NAD, 9 U/ml hexokinase (Roche Applied Science, Indianapolis, USA)] and 99 μl of water. After establishing a blind value, glucose, fructose and sucrose were measured by consecutively adding 1 U glucose-6-phosphate dehydrogenase (Roche Applied Science), 0.7 U phosphoglucone isomerase (Roche Applied Science) and 5 U invertase (Fluka, St Louis, USA). Soluble carbohydrate contents were calculated as described (Smith and Zeeman, 2006).

RNA extraction and qPCR analysis

Seeds at indicated stages were harvested into RNAlater (Sigma-Aldrich, St Louis, USA) and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, USA). For quantitative RT-PCR, RNA was treated on-column with DNase I and reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, USA). Gene-specific primers and Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, USA) were used on a 7500 Fast Real-Time PCR System (Applied Biosystems). Quantitative RT-PCR was performed using three replicates.
and ACTIN11 as a reference gene. Results were analyzed as previously described (Simon, 2003). Primers are listed in supplementary material Table S1.

**Chromatin immunoprecipitation (ChIP)**

ChIP from endosperm nuclei and seedling tissue was performed as described (Weinhofer et al., 2010). Gene-specific primers and the Fast SYBR Green Master Mix were used on the 7500 Fast Real-Time PCR System to test enrichment of H3K27me3-marked fragments. Quantitative ChIP PCR was performed using three replicates and results were analyzed as described (Simon, 2003) and are presented as percentage of input.

**Microscopy**

Seeds were harvested for periodic acid-Schiff staining at the indicated time points and incubated in 50% ethanol, 5% acetic acid and 4% formaldehyde at 4°C for 12 hours. The seeds were dehydrated in four 1-hour steps, followed by an overnight incubation in 100% ethanol. After two wash steps with 100% ethanol, the seeds were stepwise infiltrated with Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) preparation solution and incubated for 16 hours in 100% preparation solution. For embedding, seeds were mixed with polymerization solution and allowed to polymerize for at least 2 hours. The embedded seeds were sectioned into 4 µm sections using an RM2255 microtome (Leica, Wetzlar, Germany). The sections were collected on water-covered SuperFrost Plus microscope slides (Menzel, Braunschweig, Germany) and dried on a heating plate at 72°C. The sections were oxidized for 30 minutes with 1% periodic acid, washed and stained for another 30 minutes with Schiff’s reagent (Sigma-Aldrich). The sections were embedded in Entellan New Mounting Media (Electron Microscopy Sciences, Hatfield, USA) for microscopy.

Seeds were harvested at the indicated time points and stained to detect GUS activity as described (Köhler et al., 2003b). The seeds were dehydrated and embedded as described above. Clearing analysis of seeds was performed as described (Köhler et al., 2003b).

Microscopy imaging was performed using a Leica DM 2500 microscope with DIC optics. Images were captured using a Leica DFC300 FX digital camera, exported using Leica Application Suite version 2.4.0.R1 and processed using Photoshop CS5 (Adobe Systems, San Jose, USA).

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**RESULTS**

**Endosperm cellularization failure correlates with embryo arrest in fis2 and ede1 mutants**

Loss of FIS function causes an endosperm cellularization failure and arrest of embryo development, leading to seed abortion ~8 days after pollination (DAP) (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999; Köhler et al., 2003a; Guitteny et al., 2004). We addressed the question of whether arrest of embryo development upon loss of FIS function is a direct consequence of endosperm cellularization failure or rather an indirect consequence of dysregulated gene expression impacting on embryo development. If embryo arrest is a direct consequence of endosperm cellularization failure, mutants defective in endosperm cellularization are expected to have a fis-like embryo arrest phenotype. We tested this hypothesis by analyzing the ede1 mutant, which has a mutation in a plant-specific microtubule-associated protein (Pignocchi et al., 2009). Loss of EDE1 function causes endosperm cellularization failure, resulting in seeds that contain uncellularized endosperm and in embryo arrest at late heart stage (Pignocchi et al., 2009). Under our conditions, at 8 DAP 11.4% of the seeds derived from homozygous ede1-1 mutants had a fis-like phenotype, with uncellularized endosperm and embryos arrested at heart stage (n=340; Fig. 1A-C), whereas 88.6% of the seeds had cellularized endosperm and embryos progressed in their development beyond heart stage (Fig. 1E). Development of those ede1 embryos that progressed beyond heart stage was still delayed compared with wild-type seeds; at 8 DAP, ede1 mutant embryos had only reached the early torpedo stage of development, whereas wild-type embryos had already entered the bent cotyledon stage (Fig. 1E,D). Delayed embryo development was accompanied by a delay in endosperm cellularization; whereas cellularization of the peripheral endosperm was clearly visible in wild-type endosperm at 6 DAP, ede1-1 seeds reached a similar cellularization state only at ~8 DAP (Fig. 1A,E). Embryo

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**Fig. 1. Endosperm cellularization failure correlates with embryo arrest in fis2 and ede1 mutants.** Schiff-stained Technovit sections of wild-type, fis2-5 and ede1-1 Arabidopsis seeds. **(A)** Wild-type seed with embryo at torpedo stage and cellularizing endosperm at 6 DAP. **(B)** fis2-5 seed with embryo arresting at heart stage and uncellularized endosperm at 8 DAP. **(C)** ede1-1 seed with embryo arresting at heart stage and uncellularized endosperm at 8 DAP. **(D)** Wild-type seed with embryo at bent cotyledon stage and fully cellularized endosperm at 8 DAP. **(E)** ede1-1 seed with embryo at torpedo stage and cellularizing endosperm at 8 DAP. **(F)** ede1-1 seed with embryo at bent cotyledon stage and fully cellularized endosperm at 10 DAP. CV, central vacuole. Scale bar: 100 µm.
development remained delayed and *ede1* embryos reached the bent cotyledon stage 2 days later than wild-type embryos, at 10 DAP (Fig. 1F).

Together, their close correlation in *ede1* mutant seeds supports the idea that embryo arrest is a consequence of endosperm cellularization failure. It is however possible that a fraction of *ede1* mutant embryos arrest development due to a direct requirement of EDE1 function in the embryo.

**Cellularization failure is connected with increased hexose content in the endosperm**

We considered the possible consequences of cellularization failure impacting on embryo development. Endosperm cellularization causes the central vacuole to become smaller, until it becomes completely invaded by endosperm cells (Olsen, 2001). The central vacuole of the endosperm is the main storage pool for hexoses in the seed; therefore, a decrease in vacuole size in response to cellularization is likely to cause a decrease in hexose content (Morley-Smith et al., 2008). Indeed, the time of endosperm cellularization is connected with a decrease in the ratio of hexoses (glucose and fructose) to sucrose (Baud et al., 2002). Therefore, hexose levels are expected to remain high in mutants that fail to undergo endosperm cellularization.

We tested this hypothesis by measuring glucose, fructose (referred to as hexoses) and sucrose levels in *fis2* mutant seeds at 6, 7 and 8 DAP, whereby the *fis2* phenotype allowed discrimination and separate measurement of developing and arresting seeds in *fis2/+* siliques from 7 DAP onwards. Before 7 DAP, discrimination between developing and arresting *fis2* seeds was not possible, therefore we measured a mixture of 50% wild-type and 50% *fis2* seeds. At 6 DAP, hexose levels in wild type and *fis2* were comparable (Fig. 2A), correlating with the presence of a large central vacuole in wild-type seeds (Fig. 1A). At 7 DAP, the central vacuole in wild-type seeds had strongly decreased in size (supplementary material Fig. S1), correlating with decreased hexose levels (Fig. 2A). Consistent with the predictions, hexose levels in *fis2* mutant seeds remained high at 7 and 8 DAP, whereas wild-type hexose levels strongly decreased to ~25% of those present at 6 DAP. At 8 DAP, arresting *fis2* seeds contained hexose levels that were more than five times higher than those in wild-type seeds at the same time point (Fig. 2A). At 8 DAP, sucrose levels in wild-type seeds decreased to about half the amount at 6 DAP, whereas sucrose levels in *fis2* remained unchanged over the investigated time period (Fig. 2A).

The increased levels of hexoses and sucrose in *fis2* seeds at 8 DAP correlated with increased expression of the *APL3* gene, which encodes one of the large subunits of the ADP-glucose pyrophosphorylase (AGPase), a key enzyme of starch biosynthesis (Zeeman et al., 2010) (Fig. 2B-E). Expression of *APL3* is highly responsive to sucrose and glucose (Crevillen et al., 2005), making the *APL3* promoter fused to the β-GLUCURONIDASE (GUS) a suitable reporter to monitor the tissue-specific localization of carbohydrates. Whereas in *fis2* seeds at 8 DAP a strong GUS signal could be observed in the seed coat, wild-type seeds from the same siliques had only very weak GUS staining in the seed coat (Fig. 2B-E). By contrast, wild-type seeds at 5 DAP had similarly strong GUS staining in the seed coat as *fis2* seeds at 8 DAP (Fig. 2F), correlating with a similar developmental stage of the embryo.
However, whereas wild-type embryos had a pronounced GUS staining zone surrounding the embryo (Fig. 2H), this zone was absent in fis2 embryos (Fig. 2G). This staining is likely to originate from the endosperm surrounding the embryo, suggesting that the embryo receives carbohydrates from the neighboring endosperm in wild-type seeds, whereas the embryo-surrounding endosperm in fis2 fails to perform this function.

If increased hexose levels are a consequence of cellularization failure, uncellularized ede1 seeds should have similarly increased hexose levels as fis2 seeds. To test this hypothesis, we isolated ede1 seeds that failed to undergo endosperm cellularization at 8 DAP. These seeds were phenotypically clearly distinguishable from cellularized wild-type seeds by their white and glossy appearance. Uncellularized ede1 seeds had similarly increased hexose levels as uncellularized fis2 seeds (Fig. 2I), supporting the hypothesis that increased hexose levels are a consequence of cellularization failure in the endosperm.

To conclude, endosperm cellularization failure correlates with elevated hexose levels in the developing seeds, suggesting that incoming sucrose is not properly delivered to the developing embryo, causing embryo arrest.

### Restoration of cellularization by maternal loss of AGL62 normalizes fis2 seed development

If endosperm cellularization failure is the cause of embryo developmental arrest, restoration of endosperm cellularization in fis2 mutants should promote embryo development. Loss of the MADS-box transcription factor AGL62 causes precocious endosperm cellularization, implicating AGL62 as a major regulator of endosperm cellularization in *Arabidopsis* (Kang et al., 2008). In agreement with previous studies revealing prolonged expression of an AGL62 reporter construct in fis mutant endosperm (Kang et al., 2008), AGL62 transcript levels were strongly increased and prolonged in fis2 seeds compared with wild-type transcript levels, which were undetectable at 5 DAP (Fig. 3A). Reduced expression of AGL62 correlates with the onset of endosperm cellularization in *Arabidopsis* seeds (Kang et al., 2008), suggesting repression of endosperm cellularization in fis2 seeds by extended and increased AGL62 expression.

To test whether reduced dosage of AGL62 can suppress the fis2 mutant phenotype we generated fis2/+; agl62/+ double mutants. When double-heterozygous mutants were pollinated with wild-type pollen a new seed class was observed that was clearly distinguishable from arresting fis2 seeds (Fig. 3B). At 14 DAP, ~23% of the seeds were not collapsed but instead phenotypically distinguishable from wild-type seeds by their enlarged size (Fig. 3C; 25.8% aborted seeds, 23.2% non-aborted enlarged green seeds, 51% normal seeds; n=635), suggesting that agl62 could partially suppress the fis2 mutation. Although we also observed a fraction of non-collapsed seeds in fis2/+ single mutants at 14 DAP (12.2%, n=433 total seeds), none of these seeds progressed beyond the heart stage of development. By contrast, ~20% of fis2 agl62 embryos developed up to the torpedo stage (n=147 total fis2 agl62 seeds). Sections of the enlarged green fis2 agl62 seeds at 14 DAP revealed either progressing or full cellularization of the endosperm (Fig. 3D), whereby advanced levels of cellularization correlated with advanced embryo development. Development of fis2 agl62 embryos was delayed compared with wild-type embryos: whereas most wild-type embryos were in the bent cotyledon stage at 9 DAP, fis2 agl62 embryos still had a torpedo-like shape (Fig. 3D). The delayed embryo development in fis2 agl62 seeds correlated with a delay of endosperm cellularization, which occurred 4-5 days later than in wild-type seeds (Fig. 3D), suggesting that AGL62 is important but might not be the only dysregulated FIS target impacting endosperm development. Alternatively, it is possible that the paternally contributed wild-type AGL62 allele remained expressed at higher levels in fis2 seeds.

To further test the hypothesis that the agl62 mutation can suppress the fis2 mutant phenotype, we examined whether the concomitant presence of the agl62 and fis2 mutations would allow transmission of fis2 through the maternal gametophyte. fis2/+ agl62/+ mutants were pollinated with wild-type pollen and F1 seedlings derived from this cross were tested for the presence of
the fis2 mutation. Out of 34 seedlings carrying the agl62 mutation, nine were positively genotyped for the fis2 mutation, revealing ~50% transmission of the fis2 allele in the presence of the agl62 mutation. By contrast, out of 48 seedlings derived from the control cross of fis2/+ pollinated with wild-type pollen, only one seedling contained the fis2 mutation (Fig. 3C). However, this seedling was not viable and arrested development immediately after germination. We conclude that agl62 allows transmission of the fis2 allele through the female gametophyte, albeit at reduced frequency.

**Loss of maternal AGL62 normalizes triploid seed development**

Interploidy crosses of diploid maternal and tetraploid paternal plants cause increased expression of AGL62, which correlates with endosperm cellularization failure and seed abortion (Erilova et al., 2009). We tested whether maternal loss of AGL62 could suppress triploid seed failure by pollinating agl62/+ plants with diploid pollen from the omission in second division 1 (osd1) mutant. Pollination of wild-type plants with osd1 pollen leads to the formation of 100% triploid progeny (d’Erfurth et al., 2009), similar to interploidy crosses of diploid maternal and tetraploid paternal plants. Triploid seeds in the Columbia (Col) accession have similar phenotypic abnormalities as fis mutant seeds and abort containing embryos arrested at heart stage surrounded by largely uncultellurized endosperm (Dilkes et al., 2008). Upon pollination of wild-type plants with diploid osd1 pollen, 88% of the seeds were dark brown and collapsed, whereas 12% were a mixture of phenotypically normal seeds (3%) and enlarged abnormally shaped seeds that nonetheless contained developed embryos (9%) (n=461 total seeds; Fig. 4A). Pollination of agl62/+ with osd1 pollen more than doubled the number of phenotypically normal seeds and enlarged seeds (7% and 20%, respectively; n=522 total seeds; Fig. 4A-C), supporting the view that reduced levels of AGL62 can restore viable triploid seed formation. We tested the germination capacity of triploid seeds and, consistent with increased numbers of phenotypically normal and enlarged triploid seeds derived from pollinated agl62 mutant plants, the germination rate of those seeds was almost twice as high as that of wild-type triploid seeds (19% versus 36%, n=155 and 85 total seeds, respectively; Fig. 4A). Together, these findings support the hypothesis that maternal loss of AGL62 partially restores triploid seed viability.

**Restoration of endosperm cellularization is connected with decreased hexose levels**

If cellularization failure is causally connected with increased hexose levels, restoration of endosperm cellularization should be connected with decreased hexose levels. To test this hypothesis, we performed carbohydrate measurements in fis2 agl62 and fis2 seeds at 8 DAP. At this time point, fis2/+; agl62/+ double-mutant seeds cannot be distinguished from the fis2/+ seeds by eye, resulting in a mixture of 50% double-mutant and 50% fis2 seeds in the double-mutant sample. Nonetheless, in the double-mutant sample a significant reduction in soluble carbohydrate levels was observed, supporting the idea that endosperm cellularization is connected with a decrease in hexose levels (Fig. 5A). Decreased hexose levels were connected with decreased sucrose levels (Fig. 5A), suggesting that endosperm cellularization caused decreasing sink strength, as reflected by reduced sucrose levels.

Together, our data reveal that restoration of endosperm cellularization and the resulting decrease in hexose levels allow progression of embryo development, suggesting that, as a consequence of endosperm cellularization, incoming sucrose can be channeled to the embryo to support its growth. If so, we expected that fis2 embryos should survive when cultured in vitro on optimal sucrose concentrations (340 mM) (Eastmond et al., 2002). Indeed, isolated fis2 embryos had high survival rates when cultured in vitro on plates containing 340 mM sucrose (16 developing fis2 embryos out of 40 isolated embryos; Fig. 5B). Genotyping of ten surviving embryos revealed that five of them were homozygous for the fis2 mutation, four were heterozygous and one embryo was wild type, confirming that fis2 mutant embryos can indeed be rescued when cultured under appropriate conditions. By contrast, fis2 embryos did not progress in their development when cultured on low-sucrose medium (30 mM; no developing fis2 embryos out of 12 isolated embryos), similar to a failure of wild-type embryos to progress in their development when cultured on low-sucrose medium (Eastmond et al., 2002).

**AGL62 is a direct target of the FIS PRC2 complex**

Increased and prolonged expression of AGL62 in fis2 seeds suggests that AGL62 could be directly regulated by the FIS PRC2 complex. Although AGL62 is not a target of PRC2 complexes during vegetative development (Zhang et al., 2007), recent data from our laboratory revealed that the FIS PRC2 complex targets a different set of genes to PRC2 complexes during vegetative development (Weinhofer et al., 2010). Microarray profiling data of H3K27me3 localization in the endosperm revealed substantial levels of H3K27me3 at the AGL62 locus, suggesting that AGL62 is a specific FIS PRC2 target in the endosperm (Fig. 6A). We experimentally tested the presence of H3K27me3 at the AGL62 locus by ChIP experiments using purified endosperm chromatin. These experiments confirmed that AGL62 is substantially marked
by H3K27me3 in the endosperm, whereas AGL62 does not contain H3K27me3 marks in seedlings, thus rendering AGL62 an endosperm-specific PcG target gene (Fig. 6B).

If AGL62 acts directly downstream of the FIS complex, agl62 is expected to be epistatic over fis2. To test this hypothesis, we analyzed seed development in self-fertilized fis2–/+; agl62–/+ mutants. As in the agl62–/+ single mutant, ~25% of the seeds in self-fertilized fis2–/+; agl62–/+ siliques were phenotypically indistinguishable from agl62–/– seeds (23.7% early arresting seeds; n=654; \( \chi^2=0.59<\chi^2_{0.05}\mid 1=3.84; \) Fig. 6C), revealing that agl62 is epistatic over fis2. Together, these data strongly support the hypothesis that AGL62 is a downstream target gene of the FIS PRC2 complex.

**DISCUSSION**

Our study has revealed that endosperm cellularization failure in fis2 as well as edel1 mutants is closely correlated with an arrest of embryo development at the heart stage. Conversely, endosperm cellularization in a fraction of edel1 seeds correlated with generally normal, but slightly delayed, embryo development, leading to viable seeds. Endosperm cellularization failure in response to interploidy crosses also causes an arrest of embryo development at the heart stage (Scott et al., 1998), supporting the idea that cellularization of the endosperm is crucial for the embryo to proceed beyond heart stage. Endosperm cellularization in wild-type seeds was associated with reduced hexose content, which is likely to be caused by a decrease in the size of the central vacuole. Vacuolar hexoses are likely to serve as substrates for cell wall biosynthesis or, alternatively, to be channeled to the growing embryo. Embryo arrest in fis2 and edel1 mutants was accompanied by strongly increased hexose levels, correlating with a failure of the endosperm to cellularize.

The mechanism of endosperm cellularization failure is likely to differ in fis2 and edel1. Loss of FIS functions causes strong overexpression of glycosyl hydrolase-encoding genes (Weinhofer et al., 2010). Glycosyl hydrolases preferentially hydrolyse the major component of endosperm cell walls, callose (Minic and Jouanin, 2006), suggesting that repression of cell wall-degrading enzymes is a requirement for successful endosperm cellularization. This furthermore implies that endosperm cellularization failure in fis2 is caused by a failure to repress genes that encode cell wall-degrading enzymes. By contrast, EDE1 is required for microtubule organization (Pignocchi et al., 2009). Microtubules are part of the...
cell wall-forming phragmoplast, suggesting that disturbed phragmoplast formation is the cause for endosperm cellularization failure in ede1.

Despite the distinct causes of endosperm cellularization failure in each mutant, the effect on embryo development is similar, strengthening the idea that endosperm cellularization is essential for embryo development. Endosperm cellularization causes the central vacuole to decrease in size. As a corollary, incoming sucrose will no longer be predominantly channeled to the central vacuole and can instead be partitioned to the embryo, which will form the major sink in the developing seed (Morley-Smith et al., 2008). Failure of endosperm cellularization will cause the central vacuole to remain the major sink in the seed, consistent with high hexose-to-sucrose ratios in fis2 and ede1 mutant seeds. As a consequence, it is possible that sucrose is not sufficiently channeled to the embryo, but remains to be transported to the vacuole and is cleaved into hexoses. In agreement with this view, delayed endosperm cellularization in the apetala 2 mutant is similarly connected with increased hexose-to-sucrose ratios and to a delay in embryo development (Ohto et al., 2005; Ohto et al., 2009).

The tissue-specific localization of carbohydrates using the APL3 promoter supports the idea that, as a consequence of endosperm cellularization, sucrose is channeled to the embryo through the surrounding endosperm in wild-type seeds, in agreement with sucrose tracer experiments in oilseed rape (Morley-Smith et al., 2008). Consistently, APL3 expression could not be detected in the region surrounding the fis2 embryo, suggesting that owing to the lack of endosperm cellularization in fis2 the incoming sucrose remains to be channeled to the central vacuole and does not reach the embryo. Alternatively, it is possible that the embryo-surrounding endosperm in fis2 and ede1 mutants fails to appropriately differentiate to deliver incoming sucrose to the embryo. The sucrose transporter AtSUC5 is expressed in the micropylar region of the endosperm (Baud et al., 2005), indicating that this region has to achieve a specific differentiation status to deliver sucrose to the embryo. Together, we hypothesize that reduced provision of the embryo with sucrose is the likely cause for embryo arrest in mutants that fail to undergo endosperm cellularization. In line with this view is the fact that the patterning of heart stage embryos lacking FIS activity is indistinguishable from that of wild-type embryos (Leroy et al., 2007), making a defect in establishing organ identity rather unlikely as the cause for embryo arrest.

Our data further reveal that the type I MADS-box transcription factor AGL62 is a direct target gene of the FIS PRC2 complex, establishing AGL62 as an endosperm-specific PcG target gene. AGL62 is a negative regulator of endosperm cellularization (Kang et al., 2008) and our data suggest that the endosperm cellularization failure in fis2 is largely a consequence of increased AGL62 expression. The agl62 mutant is epistatic over fis2, in agreement with the view that AGL62 is acting downstream of FIS2. The fis2 phenotype could partially be reversed by maternal loss of AGL62 function. Restoration of endosperm cellularization in fis2/+ agl62/+ double-mutant seeds occurred concomitantly with embryo development, leading to the formation of viable fis2 embryos. Delayed endosperm cellularization in fis2/+ agl62/+ mutant seeds was accompanied by delayed embryo development, implying that progression of embryo development is strictly coupled to endosperm cellularization. As AGL62 is exclusively expressed in the endosperm (Kang et al., 2008), it is most likely that the normalization of fis2 embryo development in fis2/+ agl62/+ mutant seeds is a consequence of restored endosperm cellularization. Similarly, triploid seed failure could be suppressed by maternal loss of AGL62 function, in agreement with the view that endosperm cellularization failure is largely responsible for triploid seed failure (Scott et al., 1998). Loss of maternal AGL62 function has previously been shown to cause partial rescue of hybrid seeds derived from crosses of Arabidopsis thaliana and Arabidopsis arenosa (Walia et al., 2009), suggesting that increased AGL62 expression and consequently endosperm cellularization failure are common mechanisms underlying interploidy and interspecies seed failure.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. Plant J. 29, 225-235.


