Molecular Plant
Research Article

CellPress
PARTNER JOURNAL

“Love Is Strong, and You’re so Sweet”: JAGGER Is Essential for Persistent Synergid Degeneration and Polytubey Block in *Arabidopsis thaliana*

Ana Marta Pereira[1,2], Margarida Sofia Nobre[1,2,4], Sara Cristina Pinto[1,2], Ana Lúcia Lopes[1,2], Mário Luís Costa[1,2], Simona Masiero[3] and Sílvia Coimbra[1,2,*]

[1]Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

[2]Plant Functional Genomics Group, Biosystems and Integrative Sciences Institute (BioISI), 4169-007 Porto, Portugal

[3]Dipartimento di BioScienze, Università degli Studi di Milano, 20133 Milan, Italy

[4]Present address: Institute of Plant Biology and Zürich-Basel Plant Science Center, University of Zürich, Zolikerstrasse 107, 8008 Zürich, Switzerland

***Correspondence: Sílvia Coimbra (scoimbra@fc.up.pt)**

http://dx.doi.org/10.1016/j.molp.2016.01.002

Excerpt from the lyrics of ‘‘Love is Strong’’ by The Rolling Stones.

ABSTRACT

Successful double fertilization and subsequent seed development in flowering plants requires the delivery of two sperm cells, transported by a pollen tube, into the embryo sac of an ovule. The embryo sac cells tightly control synergid cell death, and as a result the polyspermy block. Arabinogalactan proteins are highly glycosylated proteins thought to be involved in several steps of the reproductive process. We show that JAGGER, Arabinogalactan Protein 4, is an important molecule necessary to prevent the growth of multiple pollen tubes into one embryo sac in *Arabidopsis thaliana*. In *jagger*, an *AGP4* knockout mutant, the pistils show impaired pollen tube blockage as a consequence of the survival of the persistent synergid. JAGGER seems to be involved in the signaling pathway that leads to a blockage of pollen tube attraction. Our results shed light on the mechanism responsible for preventing polyspermy in *Arabidopsis* and for safeguarding successful fertilization of all ovules in one pistil, ensuring seed set and the next generation.

Key words: Polytubey, persistent synergid, arabinogalactan proteins, polyspermy

Pereira A.M., Nobre M.S., Pinto S.C., Lopes A.L., Costa M.L., Masiero S., and Coimbra S. (2016). ‘‘Love Is Strong, and You’re so Sweet’’: JAGGER Is Essential for Persistent Synergid Degeneration and Polytubey Block in *Arabidopsis thaliana*. Mol. Plant. **9**, 601–614.

INTRODUCTION

In angiosperms, seed formation normally begins with the delivery of two immotile sperm cells to the embryo sac and their fusion with the two female gametes, the egg and the central cell (Russell, 1992). The sperm cells are delivered by the pollen tube. When a pollen grain lands on a compatible stigma it germinates and the tip-growing pollen tube protrudes, penetrates the pistil tissues, and continues to grow until it reaches the embryo sac (Elleman et al., 1992). In the vicinity of the embryo sac, the pollen tube is attracted by specific molecules, such as *At*Lures (Takeuchi and Higashiyama, 2012), produced by the synergid cells and released through the synergid filiform apparatus. The pollen tube grows through this apparatus, and enters the receptive synergid (Leshem et al., 2013), where its growth is arrested and it bursts, releasing the sperm cells (Hamamura et al., 2011). Afterward, the disrupted synergid degenerates and one of the sperm cells fuses with the central cell and the other sperm cell fuses with the egg cell, giving rise to the endosperm and embryo, respectively (Russell, 1992).

Little is known about the mechanisms that regulate the attraction and repulsion of pollen tubes into the ovules (Dresselhaus and Sprunck, 2012). After the egg and central cells are fertilized no more pollen tubes are attracted, thereby avoiding polytubey and, conversely, polyspermy (Beale et al., 2012). However, in the case of gamete fusion failure, the embryo sac can still attract further pollen tubes for successful fertilization (Kasahara et al., 2012). This is achieved by the maintenance of one functional (persistent) synergid. It has been shown that the fertilized female gametes independently trigger a signaling cascade, leading to the elimination of the persistent synergid and consequently blocking the production of more molecules

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and IPPE, SIBS, CAS.

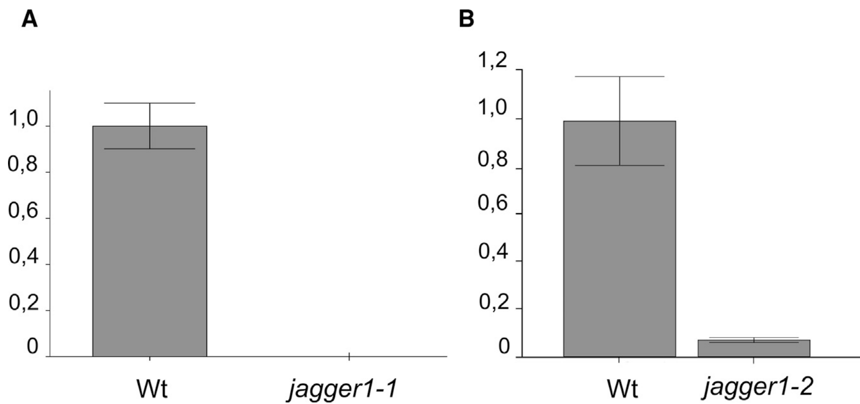

Figure 1. Relative Expression of *JAGGER* in Wild-Type, *jagger1-1*−/−, and *jagger1-2*−/− Mutant Flowers.

(A) No *JAGGER* expression was observed in *jagger1-1*−/− flower tissues.

(B) *jagger1-2*−/− had residual expression of JAGGER in flower tissues. The relative gene expression was measured using stably expressed reference genes (*RUB1* and *ACT8*) in three biological samples. Similar results were obtained for the three samples. The data correspond to the ratio of the expression in wild-type or *jagger*−/− compared with the wild-type and are the mean ± SD of three replicates of a biological sample.

for pollen tube attraction (Beale et al., 2012; Kasahara et al., 2012; Maruyama et al., 2013; Völz et al., 2013). Ethylene production guides the transduction cascade, leading to the elimination of the persistent synergid. The ethylene-insensitive mutants, *ein3* and *ein2* (*ethylene insensitive 1* and *2*), defective in the ethylene-signaling pathways, revealed the importance of ethylene for synergid cell death and the establishment of a pollen tube block (Völz et al., 2013). The central cell pathway also participates in polytubey blockage (Maruyama et al., 2013) as shown by the involvement of the fertilization-independent seed (FIS) class Polycomb Repressive Complex 2 (FIS-PRC2), which is a chromatin-modifying complex involved in gene silencing via trimethylation of lysine 27 on histone H3 (H3K27me) (Köhler et al., 2012). Recently, Maruyama et al. (2015) showed that these two pathways coordinate the elimination of the persistent synergid independently, leading to its fusion with the endosperm after fertilization and consequent dilution of the pollen tube attractants.

In all previously characterized mutants, where the fertilization of both female gametes is not accomplished, the embryo sac continues to attract more pollen tubes until it achieves successful double fertilization. However, in the mutants defective in the ethylene-signaling pathway, even when double fertilization occurs normally the embryo sac continues to attract supernumerary pollen tubes. With the exception of the involvement of ethylene and the FIS-PRC2 components, very little is known about the molecular mechanisms controlling polytubey.

Arabinogalactan proteins (AGPs) constitute a class of highly glycosylated proteins which are ubiquitous in plants. Most AGPs are predicted to be tethered to the plasma membrane by a glycosylphosphatidylinositol anchor (Oxley and Bacic, 1999; Seifert and Roberts, 2007). Accumulated evidence regarding the function and localization of AGPs suggests that they are directly or indirectly involved in the reproductive processes of many plant species (Pereira et al., 2015). Here we describe the *jagger* mutant phenotype, an *agp4* mutant, named after the rock'n'roll god Mick Jagger, who "can't get no satisfaction."

Microscopic and genetic data indicate that *jagger* is a sporophytic mutation that affects the persistent synergid cell death and thus pollen tube attraction. Double fertilization is successfully achieved when the first pollen tube bursts inside the embryo sac, pointing to a failure in the communication between the fertilized egg and/or central cell with the persistent synergid, involving the sporophytic tissues. The persistent synergid is not eliminated as expected after double fertilization, and continues to attract more pollen tubes into the embryo sac. We hypothesize that JAGGER, via its carbohydrate moiety, acts as an intermediary in the signaling pathway that triggers the persistent synergid cell elimination.

RESULTS

Isolation and Characterization of the *jagger* Mutant Lines

A Ds transposon insertion line (pst20518) from the RIKEN library and a transfer DNA (T-DNA) insertion line (GK-134A10) from the GABI-Kat library were chractereized. pst20518 contains the Ds transposon insert in the JAGGER-coding sequence, while GK-134A10 contains a T-DNA insertion in the *JAGGER* 3′ untranslated region (Supplemental Figure 1A), as confirmed by PCR and sequencing. Their homozygous lines were isolated and named as *jagger1-1*−/− and *jagger1-2*−/−, respectively. Copies of the inserts were amplified from the genomic DNA (Supplemental Figure 1B and 1C). Quantitative RT–PCR detected no *JAGGER* expression in *jagger1-1*−/− (Figure 1A), and only a residual level in *jagger1-2*−/− (Figure 1B). The *jagger1-1*−/− and *jagger1-2*−/− plants were indistinguishable from wild-type plants in vegetative growth.

To determine whether the male or the female gametophytes were affected in the mutant, reciprocal crosses were conducted between wild-type and the heterozygous mutants. As shown in Table 1, genetic analyses indicate that *jagger* male and female gametophytes transmission efficiencies are not affected.

Expression Pattern of *JAGGER* in Reproductive Tissues

The *JAGGER* promoter fused with the *GUS* (*β-glucuronidase*) reporter gene was used to determine its spatiotemporal expression pattern in the female reproductive tissues. All flowers analyzed were between stages 12 and 13 according to Smyth et al. (1990). GUS activity driven by the *JAGGER* promoter was strong in the transmitting tract cells (Figure 2A and 2C), the stigmatic cells (Figure 2B), and in the integuments close to the micropyle (Figure 2D–2G) in mature ovules. Inside the embryo sac, GUS activity was detected in the region corresponding to the synergids and egg cell localization, the egg apparatus (Figure 2D–2G). No GUS activity was detected in any other reproductive tissues. GUS activity was undetectable following fertilization (Figure 2H).

(A) *jagger1-1* transmission efficiency					
Transmission	**Cross**	**+/+**	**+/jag1**	**% Expected**	**% jag1/+**
Male	+/+ × +/−	55	65	50	54[a]
Female	+/− × +/+	63	52	50	45[b]
(B) *jagger1-2* transmission efficiency					
Transmission	**Cross**	**+/+**	**Heterozygous**	**% Expected**	**% jag2/+**
Male	+/+ × +/−	48	72	50	60[c]
Female	+/− × +/+	61	58	50	49[d]

Table 1. Ds and T-DNA Transmission Analysis of *jagger1* (A) and *jagger2* (B).

(A) The number of *jagger1-1* (+/*jag1*) and wild-type (+/+) plants among test cross progeny and the percentage of *jagger1* mutant progeny (% *jag1*/+). The transmission efficiency (TE) represents the percentage of *jagger1-1* mutant alleles successfully transmitted through male or female gametes. Chi-square test for a 1:1 segregation hypothesis was calculated.

(B) The number of *jagger1-2* (+/*jag2*) and wild-type (+/+) plants among test cross progeny and the percentage of *jagger1-2* mutant progeny (% *jag2*/+). The TE represents the percentage of *jagger1-2* mutant alleles successfully transmitted through male or female gametes. Chi-square test for a 1:1 segregation hypothesis was calculated.

[a] $\chi^2 = 0.416$ and $\chi^2_c = 3{,}841$; $0.416 < 3.841$.
[b] $\chi^2 = 0.526$ and $\chi^2_c = 3{,}841$; $0.526 < 3.841$.
[c] $\chi^2 = 0.4$ and $\chi^2_c = 3{,}841$; $0.4 < 3.841$.
[d] $\chi^2 = 0.038$ and $\chi^2_c = 3{,}841$; $0.038 < 3.841$.

In situ hybridization was performed at the same flower stages to confirm whether the GUS activity pattern obtained with $JAGGER_{prom}$:GUS reflected the real *JAGGER* expression; the presence of *JAGGER* transcripts closely correlated with GUS activity (Figure 3). Hybridization signals for the *JAGGER* antisense probe were detected throughout the stigma, style, transmitting tract, and ovule integuments as well as inside the embryo sac. No hybridization signals were detected in the reproductive tissues of the *jagger*−/− mutant.

Seed Set and Fertilization Analysis

For *jagger1-1* and *jagger1-2* mutant lines the number of viable seeds, aborted ovules, and seeds inside the siliques were analyzed, revealing no significant difference from wild-type siliques grown in the same conditions (Supplemental Figure 2). To examine whether gamete fusion was occurring normally in *jagger*−/− mutants, we used a marker line containing the construct $LAT52_{prom}$:GFP-H3.3mRFP. Plants bearing this construct produced pollen grains expressing GFP throughout the pollen grain and pollen tube, and expressed mRFP in the sperm cells. Cross-pollination experiments were carried out between *jagger1-1*−/− and *jagger1-2*−/− pistils with $LAT52_{prom}$:GFP-H3.3mRFP pollen. The crosses revealed no differences between the sperm cell fusions with the egg and the central cell in wild-type or *jagger* plants (Figure 4).

In Vivo Pollen Tube Growth Assays

Hand-pollination of emasculated wild-type pistils with *jagger*−/− pollen grains, followed by aniline blue staining, showed that the *jagger*−/− pollen tubes successfully grow through the transmitting tract cells and reach the embryo sac of the *jagger*−/− ovules accomplishing double fertilization, completing their journeys by reaching the bottom of the ovary 24 h after pollination. In the reciprocal crosses, wild-type pollen tubes also grow normally along the transmitting tract cells reaching the micropyle and entering the embryo sac *jagger*−/− pistils. In the *jagger*−/− mutant pistils, more than one pollen tube was frequently observed to enter a single embryo sac (Figure 5A and 5B). This situation is in direct contrast with observations for wild-type flowers, where each embryo sac received only one pollen tube (Figure 5C).

In *jagger*−/− no more than two pollen tubes entering the same embryo sac were ever observed. This phenomenon is called polytubey (Beale et al., 2012). To better analyze this phenotype, we calculated the number of polytubey occurrences observed in wild-type and *jagger*−/− pistils. Polytubey was observed in 18.7% ± 2.9% of *jagger1-1*−/− pistils pollinated with Nossen wild-type (Wt) pollen and 1.5% ± 0.8% of Wt pistils pollinated with jagger1-1−/− pollen grains (Student's *t*-test: $P < 0.0001$; $n = 600$ ovules for *jagger1-1*−/− × Wt; $P = 0.1257$; $n = 200$ ovules for Wt × *jagger1-1*−/−). For the second allele, polytubey was observed in 14.1% ± 1.9% of crosses between *jagger1-2*−/− pistils and Wt pollen, while in the reciprocal cross polytubey had an occurrence of 1.8% ± 1.1% (Student's *t*-test: $P < 0{,}0001$; $n = 950$ ovules for *jagger1-2*−/− × Wt; $P = 0.1149$; $n = 750$ ovules for Wt × jagger1-2−/−), as depicted in Figure 5D. The same experiment was performed for *jagger1-2*+/− pistils. Polytubey was observed in 2.13% ± 0.9% of *jagger1-2*+/− pistils pollinated with Wt pollen and 3.1% ± 1.2% of Wt pistils pollinated with *jagger1-2*+/− pollen grains (Student's *t*-test: $P = 0.1529$; $n = 750$ ovules for *jagger1-2*+/− × Wt; $P = 0.8281$; $n = 450$ ovules for Wt × *jagger1-2*+/−). The polytubey rate in the Nossen Wt self-crossed pistils was 4% ± 1.3% and in the Col-0 wild-type 3.3% ± 1%. No statistically significant differences between *jagger1-2*+/− reciprocal crosses and the wild-type were observed. The observation that the polytubey rates in heterozygous *jagger* mutant was the same as in the wild-type indicates that the defect is of sporophytic origin.

Embryo Sac Cells Develop Normally in *jagger*−/−

jagger−/− and wild-type flowers were embedded in LR-White resin, and cross sections of these inclusions were obtained. No differences were observed between the development of the *jagger*−/− and the wild-type ovules. As shown in Figure 6A and 6B, the synergids, the egg cell, and the central cell appear to develop similarly to the wild-type ovules (Figure 6D and 6E).

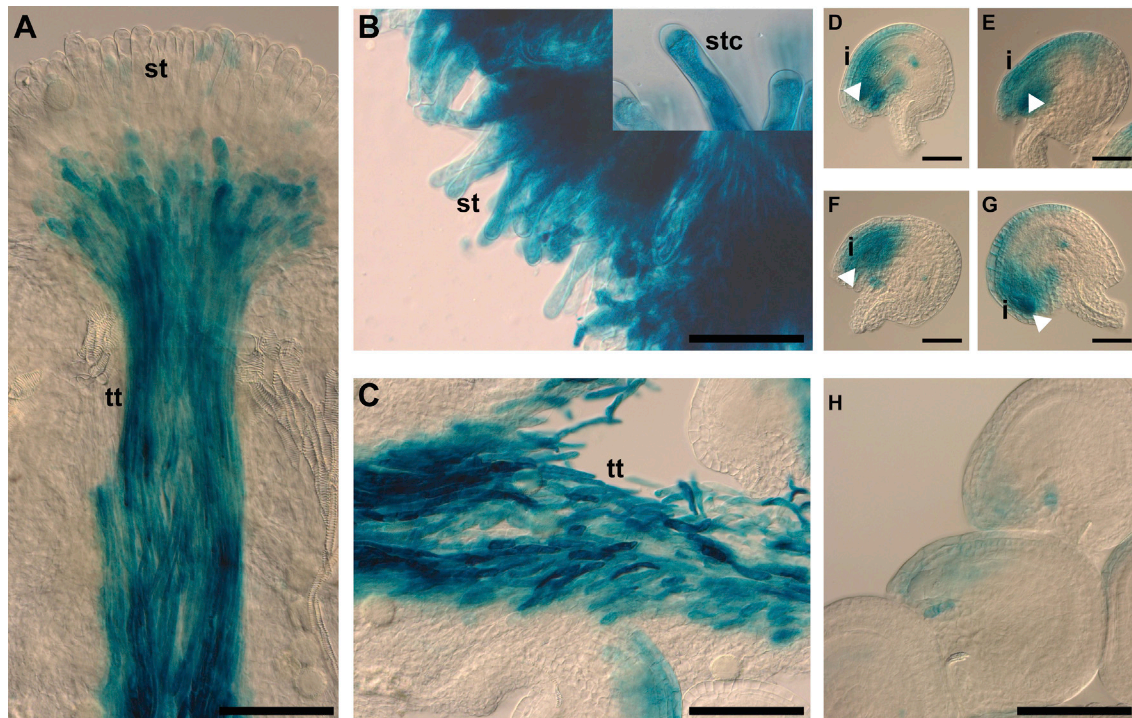

Figure 2. Histochemical Localization of GUS activity in Transgenic *Arabidopsis* Pistil Tissues Expressing the *JAGGER$_{prom}$:GUS* Fusion Gene.

(A) GUS activity driven by the JAGGER promoter is detected in the stigmatic cells (st) and the transmitting tract (tt) cells.
(B) Detail of GUS activity detected in the stigmatic cells (stc) and style cells.
(C) Strong GUS activity detected in the transmitting tract cells at higher magnification.
(D–G) GUS activity driven by JAGGER promoter in the integuments near the micropyle and inside the embryo sac in the egg apparatus (white arrowhead).
(H) Developing seeds after fertilization where GUS activity is reduced compared with the other stages of ovule development seen in **(D)**–**(G)**.
All images were obtained with a differential interference contrast (DIC) microscope. Flowers of stage 12 and stage 13 (Smyth et al., 1990) were used in this study. i, integuments; st, stigma; tt, transmitting tract. Scale bars, 100 μm **(A–C and H)** and 20 μm **(D–G)**.

Moreover, in Figure 6C it is possible to observe the entrance of a pollen tube into one of the embryo sac synergids, as well as one of the sperm cells carried inside it. To further check whether specification of the female gametophytic cells was perturbed, we crossed *jagger*−/− plants with independent transgenic plants containing the following constructs: *MYB98*$_{prom}$:GFP, At2g20595$_{prom}$:GFP, and *EC1.2*$_{prom}$:GFP. Each of the lines was crossed with *jagger*−/−, and homozygous plants generated for the marker line and T-DNA insertion mutants. No differences were observed between the control plants expressing the GFP reporter under the respective promoter and the ones crossed with *jagger*−/− (Supplemental Figure 3). *MYB98* $_{prom}$:GFP plants expressed GFP in the synergids, as did the *jagger*−/− plants crossed with this marker line (Supplemental Figure 3A and 3B). At2g20595 $_{prom}$:GFP plants showed GFP expression in the central cell; the same pattern of expression was observed in crosses with *jagger*−/− plants (Supplemental Figure 3C and 3D). GFP driven by the *EC1.2* promoter led to its expression in the egg cell, and the same expression pattern was observed in crosses with *jagger*−/− (Supplemental Figure 3E and 3F).

RNAi Suppression of *JAGGER* Driven by the *SEEDSTICK* Promoter Causes Polytubey Phenotype

We used the *SEEDSTICK* (*STK*) promoter to knock down *JAGGER* expression in the ovule-specific tissues via RNA interference (RNAi). This allowed us to verify whether the polytubey phenotype was due to the *JAGGER* expressed in the transmitting tract, integuments, and/or embryo sac. STK is ovule specific, being expressed in the integument tissues (Mizzotti et al., 2014). Transgenic plants carrying the pSTK:*JAGGER*_RNAi construct (referred to as *jagger_RNAi*) were examined. Eight *jagger_RNAi* independent lines were analyzed. By quantitative real-time RT–PCR it was observed that seven of these lines presented different levels of reduction of *JAGGER* expression (Figure 7A). For each *jagger_RNAi* line, the frequency of polytubey was calculated (Figure 7B). The reduction in *JAGGER* expression for each line correlated with an increase of polytubey frequency. The line with no reduction in JAGGER expression revealed a polytubey rate very similar to the wild-type one. This result not only shows that JAGGER present in the integuments is responsible for the observed phenotype, but also supports the sporophytic origin of the defect observed.

The *jagger* Mutation Affects Persistent Synergid Elimination

Transgenic plants carrying the 35s$_{prom}$:*JAGGER* construct were analyzed. Only three lines survived to the BASTA treatment, two of which corresponded to independent lines overexpressing the JAGGER gene, lines 1 and 2 (Supplemental Figure 4). These

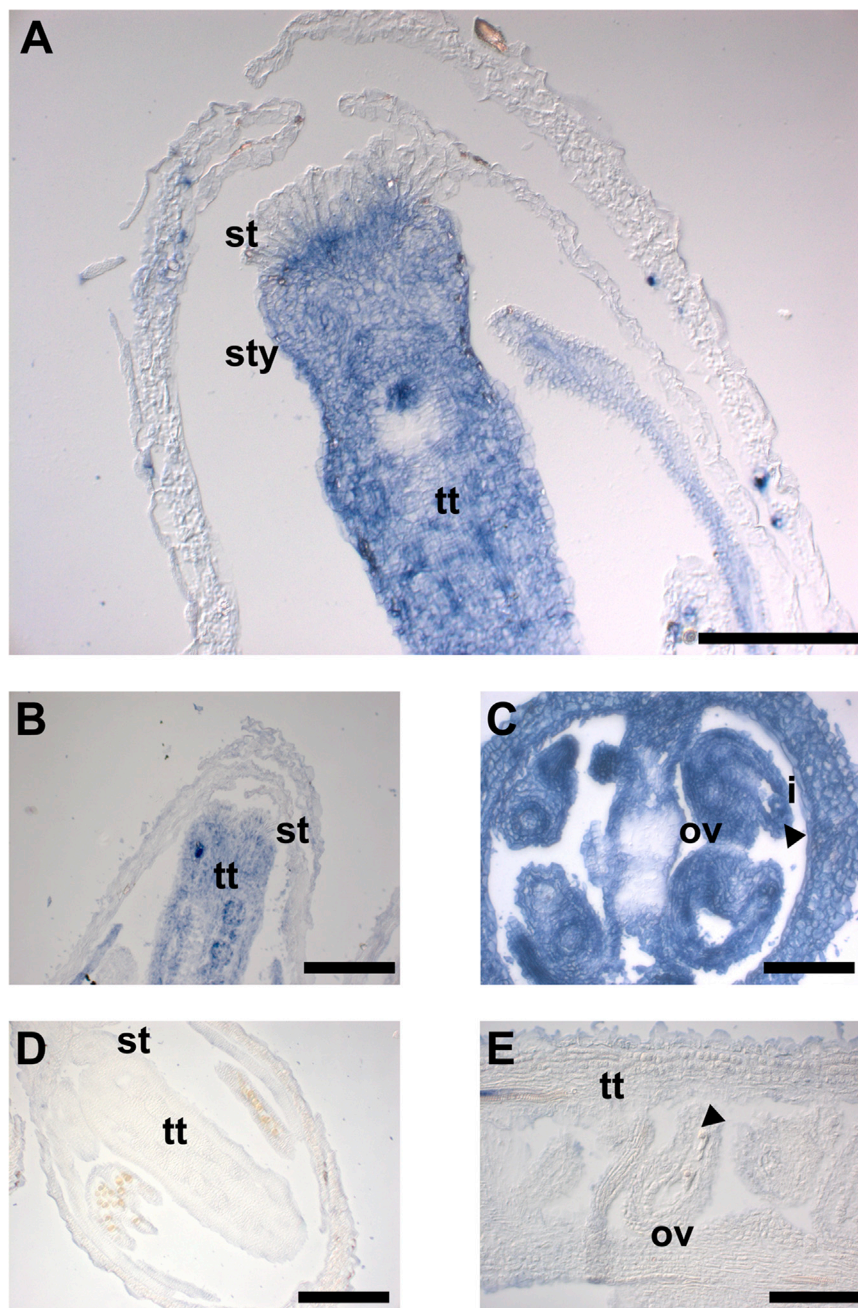

Figure 3. *JAGGER* Expression, Detected by *In Situ* Hybridization, in Sections of Wild-Type and *jagger* Pistils.

(A) Longitudinal section of a wild-type pistil revealing JAGGER expression in the transmitting tract cells of the style and the rest of the pistil.

(B) JAGGER expression in wild-type stigma and transmitting tract cells at higher magnification.

(C) A transverse section of wild-type pistil showing JAGGER expression in the ovule integuments and the egg apparatus. Arrowhead indicates micropyle.

(D and E) *jagger* pistil sections showing that JAGGER expression in the mutant pistils was below the limits of detection. Arrowhead indicates micropyle.

Flowers of stage 12 and stage 13 (Smyth et al., 1990) were used in this study. i, integuments; ov, ovule; st, stigma; sty, style; tt, transmitting tract. Scale bars, 100 μm **(A, B, D)** and 50 μm **(C, E)**.

lines exhibited normal development and their pollen grains developed normal pollen tubes when germinated on the stigma (Figure 8A). However, when aniline blue-stained pistils were checked for pollen tube growth, a considerable number of ovules remained unfertilized and callose was found to be deposited inside the embryo sacs, near the micropyle, corresponding to the location of the synergids (Figure 8D and 8E). In addition, the nucellar cells and integumentary cell near the micropyle also stained with aniline blue, indicating premature cell death (Figure 8D and 8E), although some ovules still received normally a pollen tube, as in wild-type (Figure 8B and 8C). The independent line 3, with the $35s_{prom}$:*JAGGER* construct, but without JAGGER overexpression, behaved just like the wild-type plants, and its ovules received normally a pollen tube (Figure 8F). When seed set was investigated in ethanol-fixed siliques, large areas with no seeds, most likely corresponding to aborted ovules and seeds, were detected (Figure 8G).

Given the phenotype of plants overexpressing *JAGGER*, we decided to check whether the cell death of the persisting synergid in the *jagger* mutant occurred in a similar way as that observed in wild-type plants. If the persisting synergid was alive it would still be producing pollen tube attractants, which could explain the polytubey phenotype. Analysis of cleared ovules of *jagger1-2*−/− 24 h after pollination with wild-type pollen revealed that the persisting synergid was alive even after successful fertilization and initiation of zygote formation. The presence of an extra nucleus in the micropylar region of the ovules, near the zygote, can be clearly observed (Figure 9B and 9C). In Figure 9D a persisting synergid is visible even in a six-nuclei endosperm seed. In wild-type ovules, analyzed at the same stage and pollinated with *jagger1-2*−/−, no surviving synergid nuclei could be detected in the micropylar region of the ovule (Figure 9A). Analysis of cleared ovules of *jagger1-2*+/− 24 h after pollination with wild-type pollen was also performed (Figure 9E and 9F). The absence of this extra nucleus in heterozygous *jagger*, as in wild-type pistils, indicates that the defect observed is of sporophytic origin.

Ovules of *jagger1-2*−/− flowers at the same stage and pollinated with wild-type pollen were included in LR-White resin, sectioned, and observed under a light microscope. The observations were in accordance with what was seen in the cleared ovules. A persisting synergid nucleus was visible near the micropylar region of the ovule side by side with the developing embryo (Figure 10A–10C, 10E and 10F) and zygote (Figure 10D).

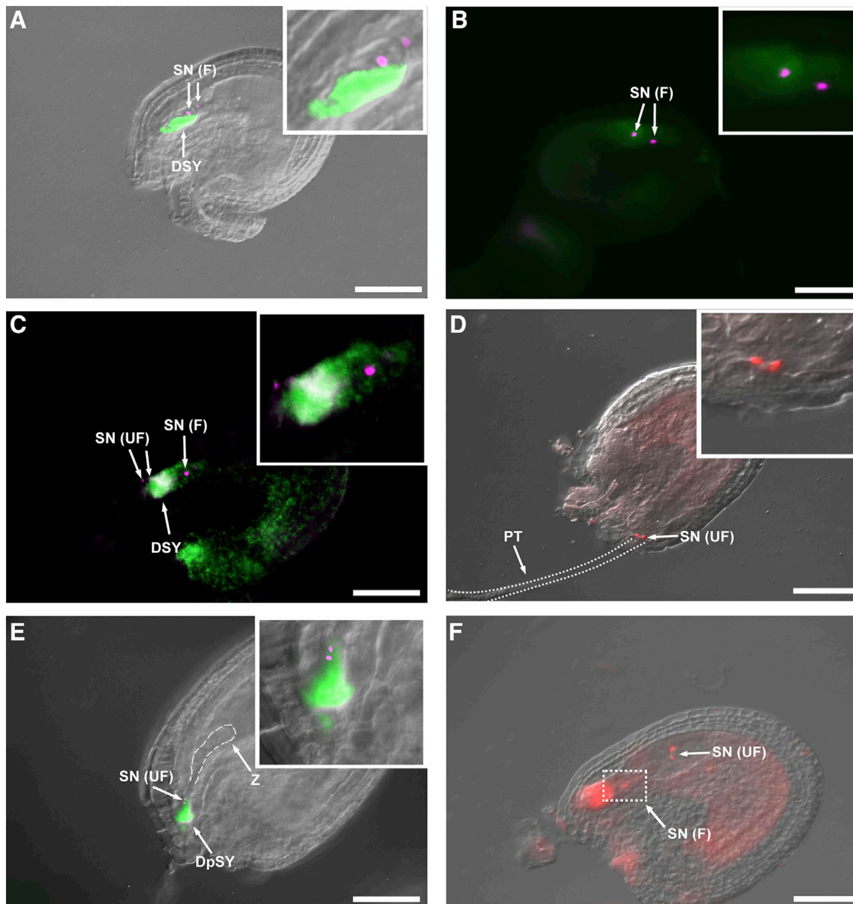

Figure 4. Crosses between *jagger*−/− Pistils and Pollen Grains Containing the Construct LAT52$_{prom}$:GFP-H3.3mRFP Reveal that Fertilization Occurs Normally but the Pollen Tube Block Is Impaired.

(A) Wild-type ovule receiving a pair of sperm nuclei (SN) represented by the magenta color and fusing (F) normally with the central and egg cell; the green signal represents the pollen tube burst inside the receptive synergid and the beginning of its degeneration (DSY, degenerating synergid).

(B) *jagger*−/− embryo sac receiving one pollen tube with a pair of SN normally released and fused with the central and egg cell.

(C) *jagger*−/− ovule receiving two pairs of sperm cells. The green signal is released from the pollen tube burst that causes degeneration of the synergid (DSY); the first SN (magenta signal) fused (F) with the egg cell and central cell nuclei. Only one fusion is visible here. The second pair of SN does not separate and persists unfused (UF).

(D) *jagger*−/− developing seed receiving a second pollen tube (PT) with a pair of SN unfused (UF) at the embryo sac entrance. The PT is in a different plane, and the dashed lines represent the PT entering the embryo sac.

(E) *jagger*−/− developing seed with a visible zygote (Z, highlighted by the dashed lines) receiving a second pair of SN unfused (UF; magenta signal); the green signal represents the burst of the PT inside the persisting synergid leading to its degeneration (DpSY).

(F) *jagger*−/− developing seed that has already received a pair of SN that fuses normally (highlighted by dashed square) and a second pair of SN unfused (UF) in the region corresponding to the developing endosperm. In **(A and D–F)** DIC and fluorescence images are overlaid. All pistils were observed 14–16 h after pollination. Scale bars, 20 μm. For improved visualization the red from RFP was replaced with magenta in images merged with green from GFP.

To prove that the extra cell in the micropylar end is not a zygote or defective zygote cell, we analyzed *jagger*−/− plants crossed with *EC1.2*$_{prom}$:GFP. This marker line, besides showing GFP expression in the egg cell, also marks the zygote and embryo after fertilization (Supplemental Figure 5A and 5D). No extra zygote was observed in the micropylar region of the ovules of *jagger*−/− crossed with *EC1.2*$_{prom}$:GFP (Supplemental Figure 5B and 5C), excluding the hypothesis of a possible extra fertilization leading to the formation of an extra zygote. Even at later stages, when the embryo starts developing, its development is similar in *EC1.2*$_{prom}$:GFP wild-type plants and *jagger*−/− × *EC1.2*$_{prom}$:GFP (Supplemental Figure 5D–5F).

To prove that this extra nucleus corresponds to the persistent synergid nucleus, we crossed *jagger*−/− with AGL62:GFP pollen (Kang et al., 2008). AGL62:GFP is known to be expressed in the endosperm but not in the embryo (Kang et al., 2008). As shown by Maruyama et al. (2015), this line can be used to analyze the persistent synergid nucleus disorganization. In this study, wild-type pistils pollinated with AGL62:GFP pollen showed ovules at the four-nuclei endosperm stage, which did not reveal any GFP signal in the persistent synergid nucleus (Supplemental Figure 6A). When crossing *jagger*−/− with this pollen donor, the GFP fluorescence was observed in the persistent synergid nucleus, at the four-nuclei endosperm stage (Supplemental Figure 6B). This indicates that the extra nucleus present at the micropylar region corresponds to the persistent synergid cell nucleus.

DISCUSSION

JAGGER Is an AGP Essential for Polytubey Block in *Arabidopsis*

Polytubey is a term used to describe the condition when an embryo sac is targeted by more than one pollen tube (Beale et al., 2012). Polytubey may result in the fusion of more than one sperm cell with a female gamete, a situation termed polyspermy. To guarantee successful seed development, the male and female gametophytes must limit the number of sperm cells delivered into the embryo sac to a single pair of sperm cells (Scott et al., 2008). Concurrently, the same gametophytes must assure fertilization of both female gametes in circumstances of sperm fusion failure with the female gametes (Kasahara et al., 2012). In several female gametophytic mutants, for example in *feronia/sirène*, where the pollen tube fails to arrest its growth and does not burst, releasing the sperm cells inside the embryo sac, ovules are capable of attracting more than one pollen tube (Huck et al., 2003; Rotman et al., 2003; Boisson-Dernier et al., 2008; Capron et al., 2008;

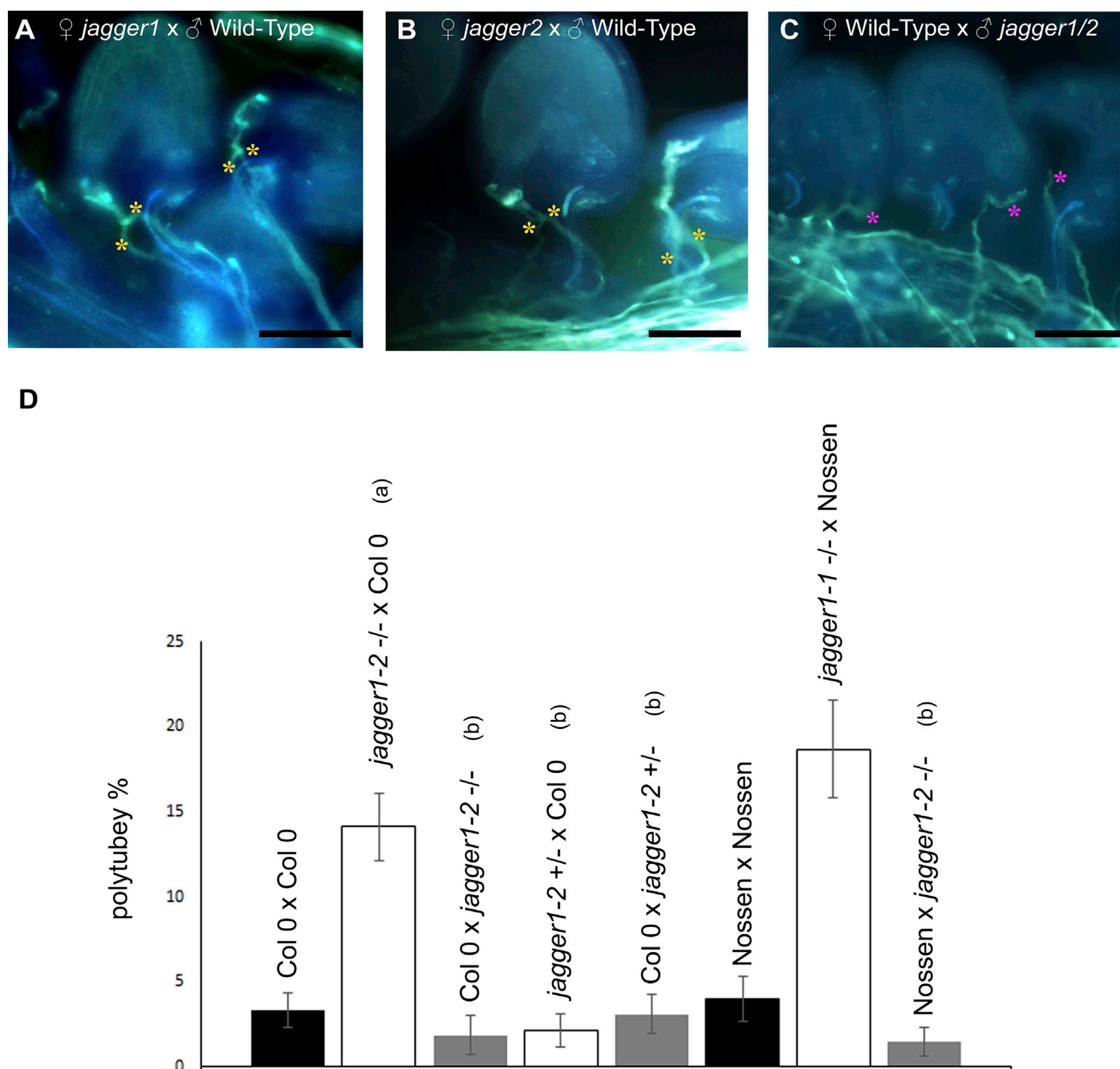

Figure 5. Aniline Blue Staining of Reciprocal Crosses between *jagger1-1/jagger1-2* and Wild-Type Flowers, and Frequency of Polytubey Observed.

(A) Cross between a female *jagger1-1*−/− flower and wild-type pollen where two polytubey occurrences can be observed as indicated by the yellow asterisks.

(B) Cross between a female *jagger1-2*−/− flower and wild-type pollen where two instances of polytubey can be observed (yellow asterisks).

(C) Cross between a female wild-type flower and *jagger1-1/1-2* pollen where normal single pollen tubes enter each ovule as pointed out by the pink asterisks.

(D) Frequency of polytubey observed in reciprocal crosses: Col-0 wild-type × Col-0 wild-type (n = 600), *jagger1-2*−/− × Col-0 wild-type (n = 950), Col-0 wild-type × *jagger1-2*−/− (n = 750), *jagger1-2*+/− × Col-0 wild-type (n = 750), Col-0 wild-type × *jagger1-2*+/− (n = 450), Nossen wild-type × Nossen wild-type (n = 600), *jagger1-1*−/− × Nossen wild-type (n = 600), Nossen wild-type (n = 200). Error bars denote mean ± SEM. a, the difference is statistically highly significant, $P < 0.05$; b, the difference is not statistically significant, $P < 0.05$.

Scale bars, 50 μm **(A–C)**.

Tsukamoto et al., 2010). Even when sperm cells are released in the embryo sac, but their fusion with the female gametes fails, as for *egg cell1* (Sprunck et al., 2012; Rademacher and Sprunck, 2013), ovules are able to attract more than one pollen tube. For the female gametophyte to block multiple pollen tubes from entering, it must ensure that double fertilization is successful and that the two sperm cells fuse with the egg and the central cell, giving rise, respectively, to the embryo and the nourishing endosperm (Beale et al., 2012). By using mutants defective in gamete fusion, *hap2* (*gcs1*) (von Besser et al., 2006), and *duo1* sperm (Durbarry et al., 2005), it was demonstrated that an embryo sac is capable of receiving up to four pollen tubes as necessary to guarantee the double fertilization, and that when this is accomplished the attraction of more pollen tubes is then blocked (Beale et al., 2012). Kasahara et al. (2012) carried out a similar study, also using the defective

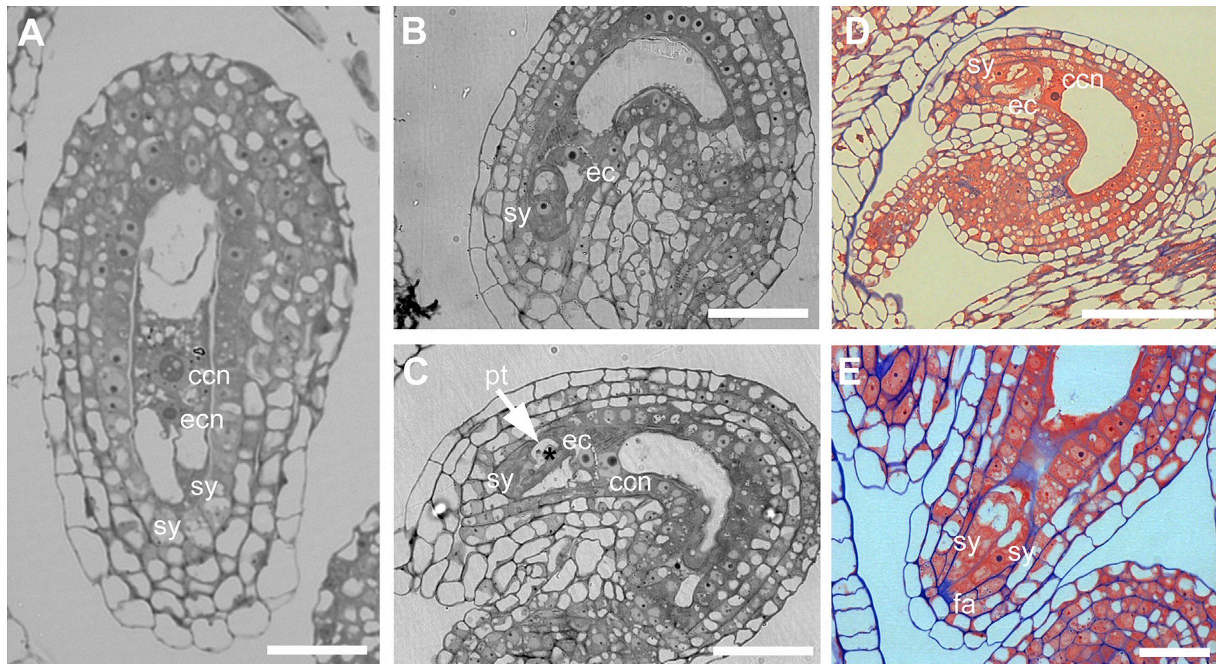

Figure 6. Cross Sections of LR-White Embedded Ovules of *jagger*−/− and Wild-Type Plants.
(A) Section of a *jagger*−/− ovule showing normal development of the female gametophytic cells central cell, egg cell, and synergids.
(B) Section of a *jagger*−/− ovule showing normal synergid and egg cell development.
(C) Section of a *jagger*−/− ovule showing a pollen tube entering the embryo sac (white arrow) and one of the sperm cell nuclei (marked with an asterisk). Normal synergid, egg, and central cell nucleus are visible.
(D) Section of a wild-type ovule showing normal development, with the central cell, the egg cell, and one of the two synergids in their common positions.
(E) Section of a wild-type ovule showing in more detail the normal development of the two synergids and their filiform apparatus.
Flowers of stage 12 and stage 13 (Smyth et al., 1990) were used in this study. ccn, central cell nucleus; ec, egg cell; ecn, egg cell nucleus; fa, filiform apparatus; sy, synergid; pt, pollen tube. Scale bars, 30 μm.

gamete fusion *hap2* (*gcs1*), *duo1*, and *duo3-2* sperm, to pollinate wild-type pistils. The primary difference between the studies was that in the latter case no more than two pollen tubes were ever observed entering one wild-type embryo sac.

The synergids are known to play a fundamental role as the source of attractants for pollen tube targeting into the embryo sac micropylar opening, as has been observed for several angiosperms including *Torenia fournieri* (Higashiyama et al., 2001; Okuda et al., 2009), *Zea mays* (Márton et al., 2005), and *A. thaliana* (Kasahara et al., 2005; Takeuchi and Higashiyama, 2012). However, other studies have also highlighted the importance of the other female gametophytic cells in pollen tube interactions with the female gametophyte; Chen et al. (2007) showed that the central cell was essential for pollen tube guidance and Scott et al. (2008) presented evidence for a polyspermy block by the egg cell but not by the central cell in *Arabidopsis*. It is now known that both the egg and the central cell have the ability to independently control the attraction of multiple pollen tubes (Maruyama et al., 2015). Several FIS class mutants such as *medea/fis1* (Grossniklaus et al., 1998; Kinoshita et al., 1999; Kiyosue et al., 1999; Luo et al., 1999), *fis2* (Chaudhury et al., 1997; Luo et al., 1999) and *fertilization-independent endosperm* (Ohad et al., 1996, 1999) display the polytubey phenotype, as shown by Maruyama et al. (2013). All these mutants belong to a class of genes coding for proteins belonging to the Polycomb Repressive Complex 2 (PRC2), a chromatin-remodeling factor responsible for gene silencing, and are shown to be involved in the blockage of polytubey by the central cell. The study of ethylene-insensitive transcription factor mutants responsible for the activation of the ethylene pathway, *ein3* and *ein2*, showed that ethylene is essential for polytubey blockage via the egg cell (Völz et al., 2013; Maruyama et al., 2015). Ethylene acts by triggering a signaling cascade, leading to the death of the persistent synergid and blocking the production of pollen tube attractants.

In the present study two independent mutant alleles were studied, *jagger1-1* and *jagger1-2*; both bearing the same defects in pollen tube attraction, the polytubey phenotype. Phenotypic analyses of *jagger* revealed no impact of the mutation on female gametophyte development. All cell types were found to be correctly specified, indicating that mutant embryo sacs reached maturity and are ready to be fertilized. In *jagger*, fertilization occurs normally, as revealed by crosses with the sperm cell marker line, $LAT52_{prom}$:GFP-H3.3mRFP, and by the correct development of embryo and endosperm in the developing seeds. The first pollen tube arrives into one of the synergids bursting and releasing a pair of sperm cells, which fuse normally with the egg and the central cell. In every case where a second pollen tube was attracted to the embryo sac, the second pair of sperm cells was observed near the micropyle or inside the already developing endosperm. Hence, in this case, similarly to what was observed for ethylene mutants, despite fertilization

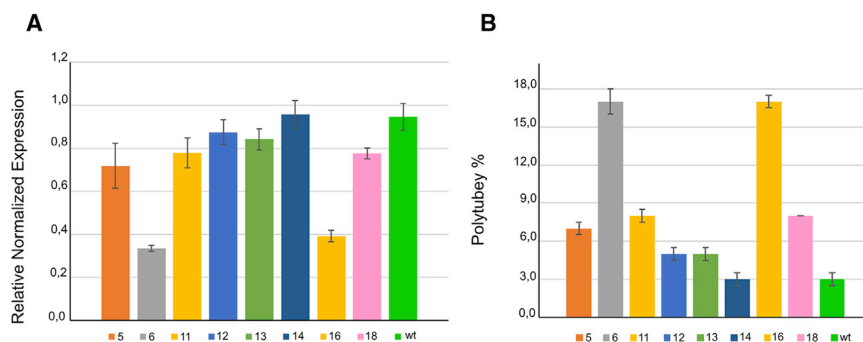

Figure 7. Analysis of *JAGGER* Expression and Polytubey Rate in pSTK:*JAGGER*_RNAi Pistils.

(A) Relative expression of *JAGGER* in wild-type and pSTK:*JAGGER*_RNAi mutant flowers. Plants 5, 6, 11, 12, 13, 14, 16, and 18 correspond to independent lines containing the pSTK:*JAGGER*_RNAi constructs. From these plants, only plants 5, 6, 11, 12, 13, 16, and 18 showed downregulation of *JAGGER* relative to the wild-type plants. Plant 14 presented levels of *JAGGER* expression similar to those of the wild-type plants. The relative gene expression was measured using stably expressed reference genes (*RUB1* and *ACT8*) in three biological samples, with similar results. The data correspond to the ratio of the expression in wild-type or pSTK:*JAGGER*_RNAi lines compared with the wild-type, and are the mean ± SD of three technical replicates of a biological sample.

(B) Frequency of polytubey observed in the eight independent lines. The increase in polytubey rate for each plant is directly correlated with a decrease in *JAGGER* expression in these plants flowers.

Wt, wild-type.

proceeding normally an additional pollen tube is attracted into the embryo sac (Völz et al., 2013).

JAGGER Plays a Role in the Elimination of the Persistent Synergid in *Arabidopsis*

According to Maruyama et al. (2015), the elimination of the second synergid occurs 12 h after pollination, by fusion with the endosperm after successful fertilization. In this work, by using the AGL62:GFP as pollen donor, we demonstrated that the persistent nucleus corresponds to the persistent synergid nucleus. In the *jagger* mutants no more than two pollen tubes were observed to enter an embryo sac, as in the case of Kasahara et al. (2012). This is most probably related to the presence of only two synergids in each *Arabidopsis* ovule. Once one synergid degenerates following the arrival of the first pollen tube, the second persisting synergid will be the source of pollen tube attractants, guiding the extra pollen tube. After invasion of this persisting synergid by a pollen tube, the production of more pollen tube attractants will be eliminated. Therefore in *jagger*, the presence of the persistent synergid long after fertilization occurs explains the attraction of the second pollen tube.

Although the polytubey phenotype is not fully penetrant in *jagger*, its presence in both mutant alleles shows that *JAGGER* disruption has consequences in polytubey block in *Arabidopsis*, acting after double fertilization, at the sporophytic level. JAGGER belongs to a family of highly glycosylated proteins, the AGPs (Seifert and Roberts, 2007). Thus, JAGGER itself or its sugar constituents may be the cause of the observed phenotype by activating a blockage system to polytubey, either through the entrance of a JAGGER-primed pollen tube into the embryo sac or by acting after fertilization, via the ethylene blocking pathway. JAGGER was already shown to be upregulated in *ctr1* (ETR1-associated protein kinase), which negatively regulates ethylene signaling, in an MPK4 (MAP kinase 4) dependent way (Brodersen et al., 2006). It is plausible to speculate that JAGGER may be acting downstream of the ethylene-signaling pathway in the polytubey blockage system, as in pistils overexpressing *JAGGER* pollen tubes could not be attracted into the embryo sacs due to premature synergid cell death.

Yet the polytubey phenotype is not fully penetrant in single mutants, *jagger1-1* and *jagger1-2*, suggesting that other genes are necessary for this function. Given the large number of genes included in the AGP family in *Arabidopsis*, some gene redundancy is expected (Kafri et al., 2009), as already found for *AGP6* and *AGP11* (Coimbra et al., 2010). Therefore, in this system the analysis of multiple mutants of related AGPs will also be important.

Both the AGP polypeptide moiety and its attached carbohydrate groups have been suggested to be important for its biological functions (Showalter, 2001; van Hengel et al., 2001). However, the mode of action of AGPs at the molecular level has not yet been elucidated. In the animal system it has long been known that several glycoproteins, such as ZP3, Zona Pellucida 3 (Han et al., 2010), or Izumo (Inoue et al., 2005; Bianchi et al., 2014), are fundamental for the fertilization process and polyspermy block. The carbohydrate moieties of AGPs are perfect candidates for involvement in these steps of sexual plant reproduction. Studies made with the Yariv reagent, which binds specifically to the β-1,3-galactoligosaccharides of AGPs (Yariv et al., 1967; Kitazawa et al., 2013), exhibit impairment in fertilization in several species such as tobacco (Qin and Zhao, 1996). Exudates produced near the ovules are enriched in glycosylated compounds (Chao, 1970; Franssen-Verheijen and Willemse, 1993). Highly glycosylated molecules such as JAGGER are attractive candidates as components of these exudates and to play a role in pollen tube growth to the ovules. In *Arabidopsis*, a mutant lacking the outer integument near the micropyle, but not the inner integuments, *inner no outer* (*ino*), fails to target the pollen tubes into the embryo sac entrance (Baker et al., 1997). Clearly the embryo sac, although essential for guidance of the pollen tubes, is not sufficient to accomplish this task. Signals from the sporophytic tissues are essential for pollen tubes to perceive ovular signaling (Palanivelu and Preuss, 2006). The expression pattern of *JAGGER* in the integuments near the micropylar entrance into the embryo, together with the polytubey phenotype observed when downregulating JAGGER under the control of STK promoter, prompt us to speculate that JAGGER or its carbohydrates are involved in priming the pollen tube before it enters the embryo sac in *A. thaliana*. This JAGGER priming is probably involved in

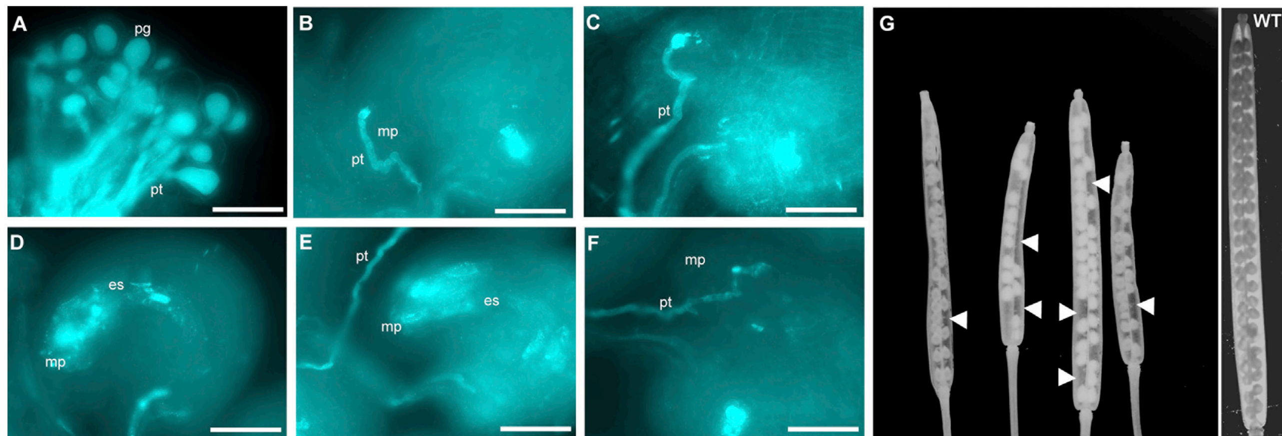

Figure 8. Aniline Blue Staining of Female Organs and Fixed Siliques of *35s_{prom}:JAGGER* Plants.

(A) Pollen grains germinating correctly on the stigma surface of a pistil from a plant overexpressing *JAGGER*. Pollen tubes grow normally through the style tissues. Only the callose stained by the aniline blue is visible.

(B) Pollen tube entering the embryo sac of a wild-type ovule.

(C) Pollen tube entering the embryo sac of a $35s_{prom}$:*JAGGER* ovule.

(D) Callose accumulation inside the embryo sac and the micropyle region of the $35s_{prom}$:*JAGGER* ovule, including the adjacent integuments.

(E) Callose accumulation inside the embryo sac and the micropyle region of a $35s_{prom}$:*JAGGER* ovule with a pollen tube passing nearby, but not entering, the ovule.

(F) Pollen tube entering the embryo sac of a pistil from a $35s_{prom}$:*JAGGER* line 3 plant (wild-type like).

(G) Ethanol-fixed siliques showing a reduced number of seeds per silique, with large areas without seeds (white arrowheads) when compared with a wild-type silique (WT).

es, embryo sac; mp, micropyle; pg, pollen grain; pt, pollen tube. Scale bars, 50 μm.

eliminating the persisting synergid, and therefore in the cessation of pollen tube attractant production, consequently avoiding polytubey.

METHODS

Plant Material and Growth Conditions

A. thaliana wild-type ecotypes Col-0 and No-0, along with two *jagger* mutant lines, were used in this work; *jagger1-1* is the RIKEN pst20518 (Ito et al., 2005) and *jagger1-2* is the GABI-Kat 34A10 line (Kleinboelting et al., 2012). The marker lines for the central cell (At2g20595$_{prom}$:GFP), egg cell (EC1.2$_{prom}$:GFP), and synergid cell (MYB98$_{prom}$:GFP) were also used (Steffen et al., 2007), as well as the marker line for the pollen tube and the sperm cells, LAT52$_{prom}$:GFP-H3.3:mRFP.

The plants were sown directly on soil and grown in an indoor growth facility with 60% relative humidity and a day/night cycle of 16 h light at 21°C and 8 h darkness at 18°C. For crosses with dehiscent anthers, closed flower buds were emasculated 24–48 h before pollination, at stage 11–12, according to Smyth et al. (1990). Gametophytic cell marker lines bearing the *jagger* mutant allele were obtained by crossing *jagger* with the At2g20595$_{prom}$:GFP, EC1.2$_{prom}$:GFP and MYB98$_{prom}$:GFP lines, and maintained by self-crossing. For PAT (phosphinotricin-acetyltransferase) selection, the seedlings were sprayed with 200 mg l^{-1} glufosinate ammonium (BASTA; Bayer Crop Science) supplemented with 0.1% (v/v) Tween 20, three or four times, every 2 days, during a 10-day period.

Genotyping

For the *jagger1-1* mutant line a PCR-based approach was employed to confirm the *Ds* insertion and identify the homozygous mutant. Genotyping primers LP-GK-134A10, RP-GK-134A10, and DS34 were used. For the *jagger1-2* mutant line the same approach was used. Genotyping primers were LP-GK-134A10, RP-GK-134A10, and 08409. All primers sequences are listed in Supplemental Table 1.

Constructs Generation and Plant Transformation

A DNA fragment encoding the promoter region of *JAGGER* was amplified by PCR using the primers AtP_4390 and AtP_4391 (Supplemental Table 1), including 3051 bp upstream of the 5′ untranslated region. The amplified fragment was cloned into pDONR207^{TM} (Invitrogen). The promoter fragment was transferred into the binary vector pBGWFS7 (Karimi et al., 2002) to obtain the *JAGGER*$_{prom}$:GUS construct. An overexpression vector was obtained using a 763-bp DNA fragment corresponding to the *JAGGER*-coding sequence. This DNA fragment was amplified by PCR using the primers AtP_4486 and AtP_4487 (Supplemental Table 1). The amplified fragment was cloned into pDONR207^{TM} (Invitrogen) and thereafter transferred into the overexpression vector pB2GW7 (Karimi et al., 2002) to obtain the 35s$_{prom}$:*JAGGER* construct. To construct pSTK:*JAGGER*_RNAi, we amplified a specific *JAGGER* fragment (231 bp) using primers AtP_4339 and AtP_4340, and recombined into RNAi vector pFGC5941 (Karimi et al., 2002) through an LR reaction (Invitrogen). The CaMV 35S promoter of the pFGC5941 vector was removed and substituted by the STK promoter (amplified using primers AtP_590 and AtP_591) (Kooiker et al., 2005). All constructs were confirmed by DNA sequencing. *A. thaliana* Col-0 was transformed by the floral dip method (Clough and Bent, 1998).

Pollen Tube Staining with Aniline Blue

Arabidopsis flowers were fixed in 10% (v/v) acetic acid in ethanol for approximately 16 h, washed three times with water and softened with 1 M NaOH for a further 16 h, washed three times with water, and incubated overnight in 0.1% (w/v) decolorized aniline blue at 4°C. The specimens were observed with a Zeiss Axiophot D1 microscope. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

Preparation of Live Plant Material for Microscopy

Emasculated pistils were observed 2 days after pollination, kept in 50 mM sodium phosphate buffer (pH 7.5) or water, and dissected under a

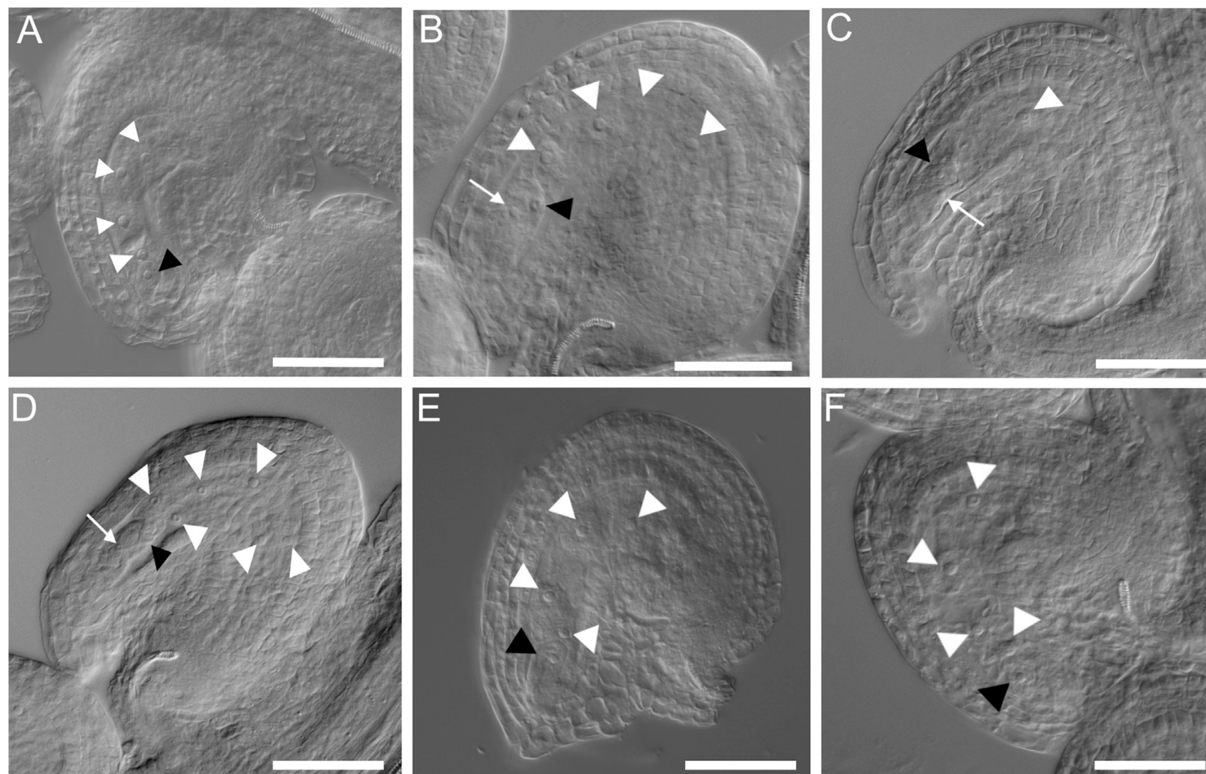

Figure 9. Cleared Whole Mounts of Wild-Type, *jagger*−/−, and jagger+/− Seeds.
(A) Wild-type developing seed with a four-nuclei endosperm (white arrowheads) and a recently formed zygote (black arrowhead).
(B) *jagger*−/− developing seed with a four-nuclei endosperm (white arrowheads), a zygote (black arrowhead), and a persisting synergid (white arrow).
(C) *jagger*−/− developing seed with two endosperm nuclei (white arrowhead, only one is visible) with a recently formed zygote (black arrowhead), and a persisting synergid (white arrow).
(D) *jagger*−/− developing seed with six visible endosperm nuclei (white arrowheads), a developing zygote (black arrowhead), and a persisting synergid (white arrow).
(E and F) *jagger*+/− with a four-nuclei endosperm (white arrowheads) and a zygote (black arrowhead).
Scale bars, 50 μm.

stereomicroscope (Model C-DSD230, Nikon) using hypodermic needles (0.4 × 20 mm; Braun). The opened carpels and the ovules that remained attached to the septum were maintained in mounting medium and covered with a coverslip. Pistils pollinated with the LAT52$_{prom}$:GFP-H3.3:mRFP pollen were observed using a Zeiss Axiophot D1 microscope (http://www.zeiss.com) equipped with differential interference contrast optics. Images were recorded using an Axiocam MRc5 camera (Zeiss) with Axiovision version 4.1.

For phenotypic characterization, ovules at different developmental stages were cleared and analyzed as described previously (Brambilla et al., 2007). Samples were observed using a Zeiss Axiophot D1 microscope equipped with differential interference contrast optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1). Aniline blue analyses of 35s$_{prom}$:JAGGER pistils were observed under an inverted microscope (Eclipse Ti-S; Nikon) and images were captured with a ProgRes MF cool monochromatic camera (Jenoptik, Jena, Germany), and processed with ProgRes CapturePro 2.8.8 software.

Preparation of Fixed Plant Material for Light Microscopy

Pistils were fixed in 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in phosphate buffer (0.025 M [pH 7], with one micro drop of Tween 80), placed under vacuum for 1 h, and incubated at 4°C overnight. After dehydration in a graded ethanol series, the material was embedded in LR-White embedding resin (London Resin Company Ltd, London, UK). Thick sections (0.5 μm) were obtained with a Leica Reichert Supernova microtome, placed on glass slides, and stained with a solution of 0.05% (w/v) toluidine blue in a phosphate-citrate buffer (pH 4–6) and counterstained with 1% (w/v) aqueous safranin O for observation by light microscopy. Bright-field observations were made on a Leica DMLB epifluorescence microscope (objectives were Leica N-Plan). Black and white images were captured with a ProgRes MF cool monochromatic camera (Jenoptik) in automatic exposure mode, and processed with ProgRes CapturePro 2.8.8 software. Color images were captured using a Zeiss Axiocam MRc3 camera using Zen Imaging Software (Zen 2011 SP1).

Siliques were fixed overnight at 4°C in 1:9 acetic acid/ethanol. The siliques were transferred to 90% ethanol for 1 h at room temperature and left in 70% ethanol at 4°C overnight. Ethanol-fixed siliques were observed under a stereomicroscope (Model C-DSD230; Nikon) and images were captured using a Zeiss Axiocam MRc3 camera using Zen Imaging Software (Zen 2011 SP1).

GUS Assays

GUS assays were performed overnight on inflorescences as described by Liljegren et al. (2000). After chemical GUS detection, the samples were incubated at 4°C overnight in clearing solution (160 g of chloral hydrate [Sigma-Aldrich] dissolved in a solution consisting of 100 ml of water and 50 ml of glycerol). A Zeiss AxioImager AZ microscope equipped with differential interference contrast optics was used to observe the assay;

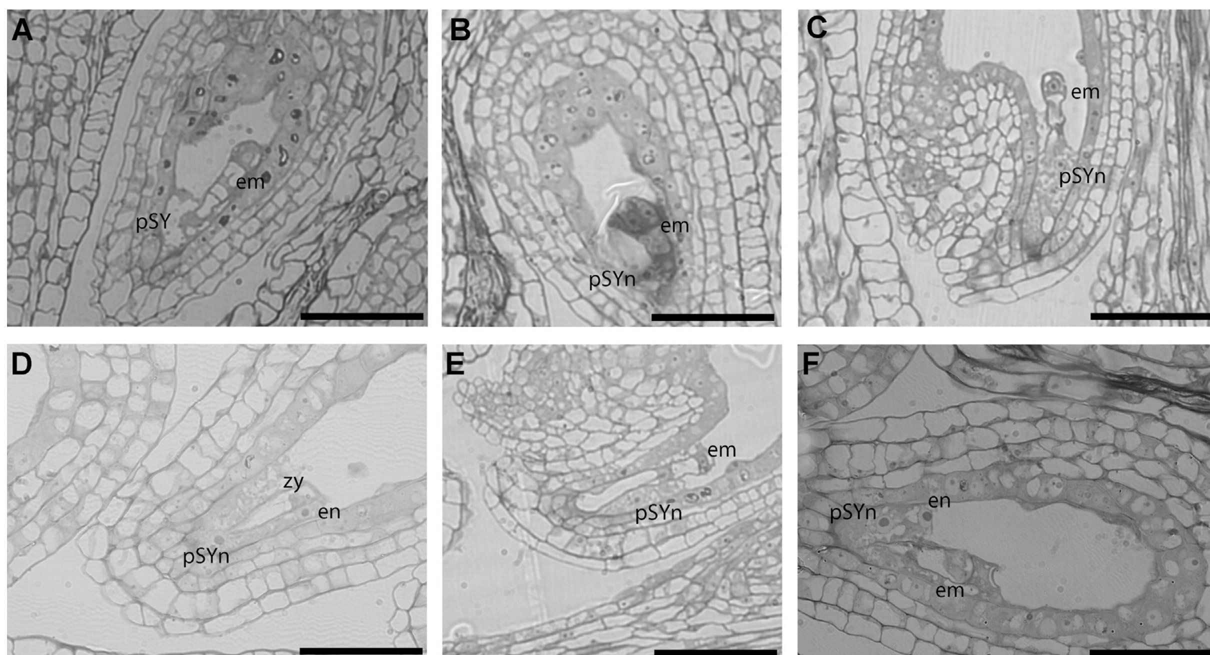

Figure 10. LR-White Cross Sections of Embedded *jagger*−/− Seeds.

(A–C, E, and F) *jagger*−/− seed with a developing embryo and a persistent synergid next to it, near the micropylar region.

(D) *jagger*−/− seed with a zygote and a persistent synergid nucleus near the micropylar end of the ovule.

en, endosperm nucleus; em, embryo; pSY, persistent synergid; pSYn, persistent synergid nucleus; zy, zygote. Scale bars, 30 μm.

images were captured with a Zeiss Axiocam MRc3 camera using Zen Imaging Software (Zen 2011 SP1).

RNA Extraction, cDNA Synthesis, and Real-Time RT–PCR

Total RNA was extracted from emasculated pistils using PureZol RNA Isolation Reagent (Bio-Rad) following the manufacturer's instructions. DNA was removed by a DNase (Thermo Scientific) treatment. The isolated RNA samples were reverse transcribed using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) and oligo(dT)$_{18}$ primers to initiate the reactions. cDNA was amplified using the SSoFast SYBR Green Supermix on an iQ5 Real-Time PCR Detection System (Bio-Rad) using the primers listed in Supplemental Table 1. Real-time RT–PCRs were run in triplicate. After 3 min at 95°C followed by a 10-s denaturation step at 95°C, samples were run for 40 cycles of 10 s at 95°C and 30 s at 60°C. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60°C to 95°C. At the end of the PCR cycles, data were analyzed with iQ5 2.0, Standard Edition Optical System Software (Bio-Rad).

In Situ Hybridization

For *in situ* hybridization analysis, *A. thaliana* flowers were fixed and embedded in paraffin as described previously. Sections of plant tissue were probed with digoxigenin-labeled *JAGGER* antisense RNA corresponding to nucleotides 35–399 (see Supplemental Table 1 for primers used for probe synthesis). Hybridization and immunological detection were performed as described by Masiero et al. (2004).

Image Processing

All images were processed for publication using ImageJ (Schneider et al., 2012) and Paint.NET (copyright dotPDN LLC and Rick Brewster).

Statistical Analysis

Data were statistically treated using GraphPad software (www.graphpad.com).

ACCESSION NUMBERS

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: *AGP4* (AT5G104380), *RUB1* (AT4G36800), *ACT8* (AT1G49240). *MYB98* (AT4G18770), *EC1.2* (AT2G21740), AT2G20595, and *STK* (AT4G09960).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

This work was financed by FEDER through the COMPETE program, and by Portuguese National funds through FCT, Fundação para a Ciência e Tecnologia (Project PTDC/AGR-GPL/115358/2009), and from an FCT PhD grant SFRH/BD/60995/2009 awarded to A.M.P. This project also benefited from financial support from COST Action FA0903: "Harnessing Plant Reproduction for Crop Improvement."

AUTHOR CONTRIBUTIONS

A.M.P., S.M., and S.C. designed the research, wrote the article, and analyzed the data. A.M.P., M.S.N., S.C.P., A.L.L., and M.L.C. performed the experiments.

ACKNOWLEDGMENTS

We thank Stefanie Sprunck for sharing with us the pollen and sperm cell marker line LAT52$_{prom}$:GFP-H3.3mRFP. We thank Gary Drews for the MYB98$_{prom}$:GFP, At2g20595$_{prom}$:GFP, EC1.2$_{prom}$:GFP, and AGL62-GFP marker lines, and Zoë Popper for comments on the manuscript. No conflict of interest declared.

Received: December 11, 2015
Revised: December 11, 2015
Accepted: January 6, 2016
Published: January 13, 2016

REFERENCES

Baker, S.C., Robinson-Beers, K., Villanueva, J.M., Gaiser, J.C., and Gasser, C.S. (1997). Interactions among genes regulating ovule development in *Arabidopsis thaliana*. Genetics **145**:1109–1124.

Beale, K.M., Leydon, A.R., and Johnson, M.A. (2012). Gamete fusion is required to block multiple pollen tubes from entering an *Arabidopsis* ovule. Curr. Biol. **22**:1090–1094.

Bianchi, E., Doe, B., Goulding, D., and Wright, G.J. (2014). Juno is the egg Izumo receptor and is essential for mammalian fertilization. Nature **508**:483–487.

Boisson-Dernier, A., Frietsch, S., Kim, T.H., Dizon, M.B., and Schroeder, J.I. (2008). The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition. Curr. Biol. **18**:63–68.

Brambilla, V., Battaglia, R., Colombo, M., Masiero, S., Bencivenga, S., Kater, M.M., and Colombo, L. (2007). Genetic and molecular interactions between BELL1 and MADS-box factors support ovule development in *Arabidopsis*. Plant Cell **19**:2544–2556.

Brodersen, P., Petersen, M., Bjørn Nielsen, H., Zhu, S., Newman, M.A., Shokat, K.M., Rietz, S., Parker, J., and Mundy, J. (2006). *Arabidopsis* MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. Plant J. **47**:532–546.

Capron, A., Gourgues, M., Neiva, L.S., Faure, J.E., Berger, F., Pagnussat, G., Krishnan, A., Alvarez-Mejia, C., Vielle-Calzada, J.P., Lee, Y.R., et al. (2008). Maternal control of male-gamete delivery in *Arabidopsis* involves a putative GPI-anchored protein encoded by the LORELEI gene. Plant Cell **20**:3038–3049.

Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **94**:4223–4228.

Chao, C.C. (1970). Further cytological studies of a periodic acid-Schiff's substance in the ovules of *Paspalum orbiculare* and *Paspalum congifiorum*. Am. J. Bot. **64**:921–930.

Chen, Y.H., Li, H.J., Shi, D.Q., Yuan, L., Liu, J., Sreenivasan, R., Baskar, R., Grossniklaus, U., and Yang, W.C. (2007). The central cell plays a critical role in pollen tube guidance in *Arabidopsis*. Plant Cell **19**:3563–3577.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16**:735–743.

Coimbra, S., Costa, M., Mendes, M.A., Pereira, A.M., Pinto, J., and Pereira, L.G. (2010). Early germination of *Arabidopsis* pollen in a double null mutant for the arabinogalactan protein genes *AGP6* and *AGP11*. Sex Plant Reprod. **23**:199–205.

Dresselhaus, T., and Sprunck, S. (2012). Plant fertilization: maximizing reproductive success. Curr. Biol. **22**:R487–R489.

Durbarry, A., Vizir, I., and Twell, D. (2005). Male germ line development in *Arabidopsis*: duo pollen mutants reveal gametophytic regulators of generative cell cycle progression. Plant Physiol. **137**:297–307.

Elleman, C.J., Franklin-Tong, V., and Dickinson, H.G. (1992). Pollination in species with dry stigmas: the nature of the early stigmatic response and the pathway taken by pollen tubes. New Phytol. **60**:413–424.

Franssen-Verheijen, A.A.W., and Willemse, M.T.M. (1993). Micropylar exudate in Gasteria (Aloaceae) and its possible function in pollen tube growth. Am. J. Bot. **80**:253–262.

Grossniklaus, U., Vielle-Calzada, J.P., Hoeppner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by MEDEA, a polycomb group gene in *Arabidopsis*. Science **280**:446–450.

Hamamura, Y., Saito, C., Awai, C., Kurihara, D., Miyawaki, A., Nakagawa, T., Kanaoka, M.M., Sasaki, N., Nakano, A., Berger, F., et al. (2011). Live-cell imaging reveals the dynamics of two sperm cells during double fertilization in *Arabidopsis thaliana*. Curr. Biol. **21**:497–502.

Han, L., Monné, M., Okumura, H., Schwend, T., Cherry, A.L., Flot, D., Matsuda, T., and Jovine, L. (2010). Insights into egg coat assembly and egg-sperm interaction from the X-ray structure of full-length ZP3. Cell **143**:404–415.

Higashiyama, T., Yabe, S., Sasaki, N., Nishimura, Y., Miyagishima, S., Kuroiwa, H., and Kuroiwa, T. (2001). Pollen tube attraction by the synergid cell. Science **293**:1480–1483.

Huck, N., Moore, J.M., Federer, M., and Grossniklaus, U. (2003). The *Arabidopsis* mutant *feronia* disrupts the female gametophytic control of pollen tube reception. Development **130**:2149–2159.

Inoue, N., Ikawa, M., Isotani, A., and Okabe, M. (2005). The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature **434**:234–238.

Ito, T., Motohashi, R., Kuromori, T., Noutoshi, Y., Seki, M., Kamiya, A., Mizukado, S., Sakurai, T., and Shinozaki, K. (2005). A resource of 5,814 dissociation transposon-tagged and sequence-indexed lines of *Arabidopsis* transposed from start loci on chromosome 5. Plant Cell Physiol. **46**:1149–1153.

Kafri, R., Springer, M., and Pilpel, Y. (2009). Genetic redundancy: new tricks for old genes. Cell **136**:389–392.

Kang, I.H., Steffen, J.G., Portereiko, M.F., Lloyd, A., and Drews, G.N. (2008). The AGL62 MADS domain protein regulates cellularization during endosperm development in *Arabidopsis*. Plant Cell **20**:635–647.

Karimi, M., Inzé, D., and Depicker, A. (2002). GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. **7**:193–195.

Kasahara, R.D., Portereiko, M.F., Sandaklie-Nikolova, L., Rabiger, D.S., and Drews, G.N. (2005). MYB98 is required for pollen tube guidance and synergid cell differentiation in *Arabidopsis*. Plant Cell **17**:2981–2992.

Kasahara, R.D., Maruyama, D., Hamamura, Y., Sakakibara, T., Twell, D., and Higashiyama, T. (2012). Fertilization recovery after defective sperm cell release in *Arabidopsis*. Curr. Biol. **22**:1084–1089.

Kinoshita, T., Yadegari, R., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Imprinting of the MEDEA Polycomb gene in the *Arabidopsis* endosperm. Plant Cell **11**:1945–1952.

Kitazawa, K., Tryfona, T., Yoshimi, Y., Hayashi, Y., Kawauchi, S., Antonov, L., Tanaka, H., Takahashi, T., Kaneko, S., Dupree, P., et al. (2013). β-Galactosyl Yariv reagent binds to the β-1,3-galactan of arabinogalactan proteins. Plant Physiol. **161**:1117–1126.

Kiyosue, T., Ohad, N., Yadegari, R., Hannon, M., Dinneny, J., Wells, D., Katz, A., Margossian, L., Harada, J.J., Goldberg, R.B., et al. (1999). Control of fertilization-independent endosperm development by the MEDEA Polycomb gene in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **96**:4186–4191.

Kleinboelting, N., Huep, G., Kloetgen, A., Viehoever, P., and Weisshaar, B. (2012). GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. Nucleic Acids Res. **40**:D1211–D1215.

Köhler, C., Wolff, P., and Spillane, C. (2012). Epigenetic mechanisms underlying genomic imprinting in plants. Annu. Rev. Plant Biol. **63**:331–352.

Kooiker, M., Airoldi, C.A., Losa, A., Manzotti, P.S., Finzi, L., Kater, M.M., and Colombo, L. (2005). BASIC PENTACYSTEINE1, a GA binding protein that induces conformational changes in the regulatory region of the homeotic *Arabidopsis* gene SEEDSTICK. Plant Cell **17**:722–729.

Leshem, Y., Johnson, C., and Sundaresan, V. (2013). Pollen tube entry into the synergid cell of *Arabidopsis* is observed at a site distinct from the filiform apparatus. Plant Reprod. **26**:93–99.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L., and Yanofsky, M.F. (2000). SHATTERPROOF MADS-box genes control seed dispersal in *Arabidopsis*. Nature **404**:766–770.

Luo, M., Bilodeau, P., Koltunow, A., Dennis, E.S., Peacock, W.J., and Chaudhury, A.M. (1999). Genes controlling fertilization-independent seed development in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **96**:296–301.

Márton, M.L., Cordts, S., Broadhvest, J., and Dresselhaus, T. (2005). Micropylar pollen tube guidance by *egg apparatus 1* of maize. Science **307**:573–576.

Maruyama, D., Hamamura, Y., Takeuchi, H., Susaki, D., Nishimaki, M., Kurihara, D., Kasahara, R.D., and Higashiyama, T. (2013). Independent control by each female gamete prevents the attraction of multiple pollen tubes. Dev. Cell. **25**:317–323.

Maruyama, D., Völz, R., Takeuchi, H., Mori, T., Igawa, T., Kurihara, D., Kawashima, T., Ueda, M., Ito, M., Umeda, M., et al. (2015). Rapid elimination of the persistent synergid through a cell fusion mechanism. Cell **161**:907–918.

Masiero, S., Li, M.A., Will, I., Hartmann, U., Saedler, H., Huijser, P., Schwarz-Sommer, Z., and Sommer, H. (2004). INCOMPOSITA: a MADS-box gene controlling prophyll development and floral meristem identity in *Antirrhinum*. Development **131**:5981–5990.

Mizzotti, C., Ezquer, I., Paolo, D., Rueda-Romero, P., Guerra, R.F., Battaglia, R., Rogachev, I., Aharoni, A., Kater, M.M., Caporali, E., et al. (2014). SEEDSTICK is a master regulator of development and metabolism in the *Arabidopsis* seed coat. PLoS Genet. **10**:e1004856.

Ohad, N., Margossian, L., Hsu, Y.-C., Williams, C., Repetti, P., and Fischer, R.L. (1996). A mutation that allows endosperm development without fertilization. Proc. Natl. Acad. Sci. USA **93**:5319–5324.

Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Mutations in FIE, a WD Polycomb group gene, allow endosperm development without fertilization. Plant Cell **11**:407–416.

Okuda, S., Tsutsui, H., Shiina, K., Sprunck, S., Takeuchi, H., Yui, R., Kasahara, R.D., Hamamura, Y., Mizukami, A., Susaki, D., et al. (2009). Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature **458**:357–361.

Oxley, D., and Bacic, A. (1999). Structure of the glycosylphosphatidylinositol anchor of an arabinogalactan protein from *Pyrus communis* suspension-cultured cells. Proc. Natl. Acad. Sci. USA **96**:14246–14251.

Palanivelu, R., and Preuss, D. (2006). Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro. BMC Plant Biol. **6**:7.

Pereira, A.M., Pereira, L.G., and Coimbra, S. (2015). Arabinogalactan proteins: rising attention from plant biologists. Plant Reprod. **28**:1–15.

Qin, Y., and Zhao, J. (1996). Localization of arabinogalactan proteins in egg cells, zygotes, and two-celled proembryos and effects of β-d-glucosyl Yariv reagent on egg cell fertilization and zygote division in *Nicotiana tabacum* L. J. Exp. Bot. **57**:2061–2074.

Rademacher, S., and Sprunck, S. (2013). Downregulation of egg cell-secreted EC1 is accompanied with delayed gamete fusion and polytubey. Plant Signal. Behav. **8**:e27377.

Rotman, N., Rozier, F., Boavida, L., Dumas, C., Berger, F., and Faure, J.E. (2003). Female control of male gamete delivery during fertilization in *Arabidopsis thaliana*. Curr. Biol. **13**:432–436.

Russell, S.D. (1992). Double fertilization. Int. Rev. Cytol. **140**:357–388.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods **9**:671–675.

Scott, R.J., Armstrong, S.J., Doughty, J., and Spielman, M. (2008). Double fertilization in *Arabidopsis thaliana* involves a polyspermy block on the egg but not the central cell. Mol. Plant **4**:611–619.

Seifert, G.J., and Roberts, K. (2007). The biology of arabinogalactan proteins. Annu. Rev. Plant Biol. **58**:137–161.

Showalter, A.M. (2001). Arabinogalactan-proteins: structure, expression and function. Cell. Mol. Life Sci. **58**:1399–1417.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis*. Plant Cell **2**:755–767.

Sprunck, S., Rademacher, S., Vogler, F., Gheyselinck, J., Grossniklaus, U., and Dresselhaus, T. (2012). Egg cell-secreted EC1 triggers sperm cell activation during double fertilization. Science **338**:1093–1097.

Steffen, J.G., Kang, I.-H., Macfarlane, J., and Drews, G.N. (2007). Identification of genes expressed in the *Arabidopsis* female gametophyte. Plant J. **51**:281–292.

Takeuchi, H., and Higashiyama, T. (2012). A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in *Arabidopsis*. PLoS Biol. **10**:e1001449.

Tsukamoto, T., Qin, Y., Huang, Y., Dunatunga, D., and Palanivelu, R. (2010). A role for LORELEI, a putative glycosylphosphatidylinositol anchored protein, in *Arabidopsis thaliana* double fertilization and early seed development. Plant J. **62**:571–588.

van Hengel, A.J., Tadesse, Z., Immerzeel, P., Schols, H., Van Kammen, A., and de Vries, S.C. (2001). N-Acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. Plant Physiol. **125**:1880–1890.

Völz, R., Heydlauff, J., Ripper, D., von Lyncker, L., and Groß-Hardt, R. (2013). Ethylene signaling is required for synergid degeneration and the establishment of a pollen tube block. Dev. Cell. **25**:310–316.

von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). *Arabidopsis* HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. Development **133**:4761–4769.

Yariv, J., Lis, H., and Katchalski, E. (1967). Precipitation of arabic acid and some seed polysaccharides by glycosylphenylazo dyes. Biochem. J. **105**:1C–2C.