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GEX3, Expressed in the Male Gametophyte and in the Egg Cell of *Arabidopsis thaliana*, Is Essential for Micropylar Pollen Tube Guidance and Plays a Role during Early Embryogenesis

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ABSTRACT Double fertilization in flowering plants occurs when the two sperm cells, carried by the pollen tube, are released in a synergid cell of the embryo sac and then fertilize the egg and the central cell. Proteins on the surfaces of the sperm, egg, central, and synergid cells might be important for guidance and recognition/fusion of the gametes. Here, we present functional analyses of *Arabidopsis GEX3*, which encodes a plasma membrane-localized protein that has homologs in other plants. *GEX3* is expressed in both the vegetative and sperm cells of the male gametophyte and in the egg cell of the female gametophyte. Transgenic lines in which *GEX3* was down-regulated or overexpressed, using the *Arabidopsis GEX2* promoter, had reduced seed set. Reciprocal crosses and imaging after pollination with a reporter line showed that, in both cases, the defect causing reduced seed set occurred in the female. In the antisense lines, micropylar pollen tube guidance failed. In the overexpression lines, fertilization of mutant ovules was mostly blocked because pollen tube guidance failed, although, occasionally, non-viable embryos were formed. We conclude that properly regulated expression of GEX3 in the egg cell of *Arabidopsis* is essential for pollen tube guidance.

Key words: antisense; double fertilization; female gametophyte; real-time PCR; plasma membrane protein; PQQ domain.

INTRODUCTION

In flowering plants, the male gametophyte (pollen grain) contains three cells: a large vegetative cell, which forms the pollen tube, and two sperm cells enclosed within the vegetative cell cytoplasm (McCormick, 2004). The female gametophyte (embryo sac) develops within the ovule and consists of seven cells of four types: three antipodal cells, two synergid cells, one egg cell, and one central cell (Yadegari and Drews, 2004). The fertilization process starts when pollen grains land on female stigmatic cells, where they hydrate and begin germination. After pollen tubes exit the stigma cell wall, they enter the transmitting tract and grow until they turn towards an available ovule, grow along the funiculus, and reach the micropyle (Higashiyama, 2002; Iwakawa et al., 2006).

Pollen tube guidance can be divided into two phases: the sporophytic phase, for which several extracellular pollen guidance signals in the transmitting tract have been reported (Wu et al., 2000; Johnson and Preuss, 2002; Palanivelu et al., 2003), and the gametophytic phase, which depends on signals derived from the female gametophyte (Hulskamp et al., 1995; Higashiyama et al., 2003), and is also divided into two phases: funicular and micropylar guidance (Hulskamp et al., 1995; Higashiyama et al., 2003; Weterings and Russell, 2004). The two synergid cells within the female gametophyte play an essential role during pollen tube guidance, as shown in *Torenia fournieri* (Higashiyama et al., 2001) and in the *Arabidopsis mma1* and *maa3* mutants (Shimizu and Okada, 2000). Upon arrival at the embryo sac, the pollen tube enters through the filiform apparatus, formed by the cell wall of the two synergid cells. One of the two synergid cells initiates cell death, followed by pollen tube reception (Sandaklie-Nikolova et al., 2007) and discharge of the two sperm cells. In the *Arabidopsis gfa2* mutant, which fails to undergo synergid cell death upon pollen tube arrival to the micropyle, fertilization does not occur (Christensen et al., 2002). Furthermore,

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the receptor-like kinase Feronia, expressed in the filiform apparatus, was shown to be a component in the signaling pathway involved in pollen tube reception (Escobar-Restrepo et al., 2007), supporting a key role for the synergid cells during fertilization.

However, the mechanism of pollen tube guidance within the female gametophyte is not fully understood. A secreted protein located in the maize egg apparatus, EA1, (Marton et al., 2005), and two *Arabidopsis* proteins involved in synergid cell development, MAA1 and MAA3 (Shimizu and Okada, 2000), affect gametophytic pollen tube guidance, while a protein expressed specifically in the sperm cells and located at the plasma membrane, GCS1/HAP2, plays a role in micropylar pollen tube guidance (von Besser et al., 2006). Recently, a nuclear protein expressed in the central cell, CCG, was shown to be necessary for pollen tube guidance (Chen et al., 2007). To date, no plasma membrane-localized proteins in the egg cell have been implicated in pollen tube guidance.

Here, we present *Arabidopsis GEX3* (*At5g16020*), encoding a protein of unknown function that we show is located at the plasma membrane. The promoter of *GEX3* directs expression of a reporter gene in the vegetative and sperm cells within the male gametophyte and in the egg cell within the female gametophyte. We generated transgenic lines down-regulating or overexpressing *Arabidopsis GEX3*; these lines had reduced seed set. These phenotypes were characterized using reciprocal crosses with WT plants and with a *ProLAT52:GUS* reporter line, by *in vivo* pollen tube growth assays, and by quantification of *GEX3* mRNA levels using real-time PCR. We found that when *GEX3* was down-regulated in the female gametophyte, the pollen tubes turned towards the ovule on the funiculus but failed to reach the micropyle. When *GEX3* was overexpressed in the female gametophyte, fertilization of mutant ovules was mostly blocked because pollen tube guidance failed, although a few non-viable embryos were formed in those overexpressing ovules that attracted pollen tubes. These results show that *Arabidopsis* GEX3 is important for micropylar pollen tube guidance and that it also plays a role during early embryogenesis.

RESULTS

GEX3 Is Conserved in Plants and Contains PQQ Domains

We previously identified *GEX1* (for *Gamete-expressed 1*) and *GEX2* as sperm-expressed genes in *Arabidopsis* (Engel et al., 2005). To identify other potentially sperm-specific genes for functional analyses, we first compared maize sperm cell ESTs (Engel et al., 2003) to maize ESTs from other tissues, using the TIGR database (www.tigr.org). We found two overlapping ESTs, only present in the sperm cell library, that fulfilled our search criteria, and so we designated the corresponding gene *GEX3*. We identified a full-length version of maize *GEX3* in the MAGI database (http://magi.plantgenomics.iastate.edu). Using maize *GEX3* as a query yielded few matches in databases—the only ESTs were from cDNA libraries prepared from *Oryza sativa* sperm cells and *Plumbago zeylanica* sperm cells, and from mixed reproductive tissues of *Cirsium arvense*; there were also matches to genomic DNA sequences of Sorghum, Poplar, Selaginella, Cleome, and *Arabidopsis*. GEX3 in *Arabidopsis* (At5g16020) is annotated as a hypothetical protein of unknown biological function. Figure 1A shows the gene structure of *Arabidopsis GEX3*. Figure 1B shows the beta-propeller repeats (PQQ domains) found at the N-terminus of GEX3 proteins, as well as the predicted signal peptide and the predicted transmembrane domain. The PQQ domain is a beta-propeller repeat occurring in enzymes that use pyrrolo–quinoline quinone (PQQ) as a cofactor; examples include Ire1p-like Ser/Thr kinases (Koizumi et al., 2001) and prokaryotic dehydrogenases (Keitel et al., 2000). Although there are no sequence similarities in the primary structures of quinoproteins with PQQ domains, all these proteins have a common structure, called a 'propeller fold', which is composed of antiparallel β-sheets arranged with radial symmetry, so that the PQQ

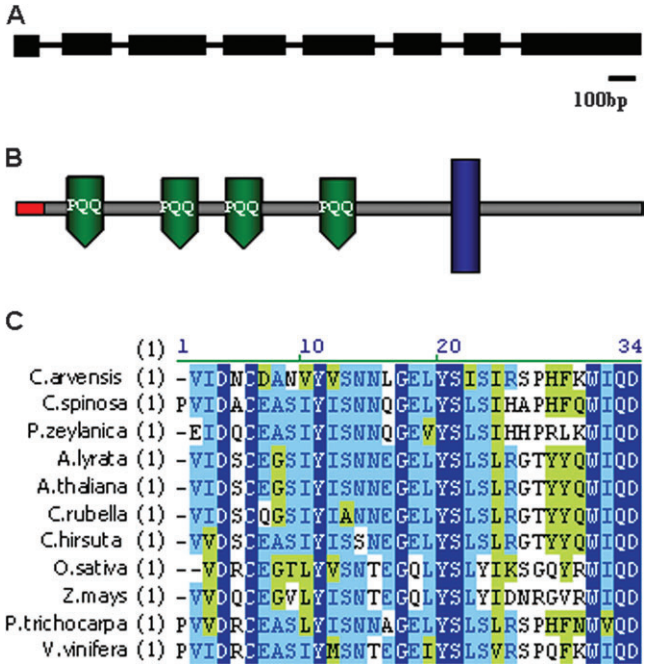

Figure 1. Gene Structure of *GEX3* and Protein Features of GEX3.
(A) Gene diagram of the genomic sequence of *Arabidopsis GEX3*.
(B) Diagram of protein domains in GEX3. The predicted signal peptide is in red, the predicted PQQ domains (β-propeller repeats) in green, and the predicted transmembrane domain in blue.
(C) Amino acid sequence alignment of one of the PQQ domains, from 11 species: *Cirsium arvensis*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Cardamine hirsuta*, *Capsella rubella*, *Cleome spinosa*, *Oryza sativa*, *Poplar trichocarpa*, *Plumbago zeylanica*, *Vitis vinifera*, and *Zea mays*. Identical amino acids are highlighted in dark blue, conserved amino acids in pale blue, and amino acids of the same family in green.

cofactor would be located at the center (Matsushita et al., 2002). PQQ domains are similar to WD-repeats, found in all eukaryotes and known to create a stable platform that allows reversible interactions with other proteins (Smith et al., 1999). Figure 1C shows an amino acid alignment of one of the PQQ domains of GEX3 in 11 plant species. Nine residues are identical within the 34 amino acid domain. Figure S1 shows the amino acid sequence alignment for GEX3 in six plant species. GEX3 is predicted to be located at the plasma membrane, with the N-terminus predicted to be extracellular and the C-terminus predicted to be cytoplasmic. Most of the amino acids that are identical among the six species are located in the N-termini.

GEX3 Has Not Evolved under Positive Selection in the Brassicaceae

Genes that encode proteins involved in reproduction, in both animals and plants, are sometimes under positive selection (Wyckoff et al., 2000; Begun et al., 2007). An indication of positive selection is a higher non-synonymous (K_A; amino acid replacing) than synonymous (K_S; silent) substitution rate (Li, 1997; Swanson et al., 2001). The ratio of the two rates, K_A/K_S, measures the magnitude and direction of selective pressure on a protein, with $K_A/K_S = 1$, < 1, and > 1, indicating neutral evolution, purifying selection, or positive diversifying selection, respectively (Li, 1997; Swanson et al., 2001). To determine whether *GEX3* might be subject to positive selection, we sequenced portions of the genomic sequence of *GEX3* from *Arabidopsis lyrata*, which is 5 000 000 years apart from *Arabidopsis thaliana* (Al-Shehbaz and O'kane, 2002), as well as from two more distant relatives, *Cardamine hirsuta* and *Capsella rubella*, which are 14 000 000 and 20 000 000 years apart from *Arabidopsis thaliana*, respectively (Rossberg et al., 2001; Hay and Tsiantis, 2006). We carried out sliding window analyses along the nucleotide sequence, using the software dnasp (www.ub.es/dnasp/) and the same parameters as in Escobar-Restrepo et al. (2007). None of the pairs of DNA sequences analyzed (i.e. *A. thaliana* vs *A. lyrata*, *A. thaliana* vs *C. hirsuta* or *A. thaliana* vs *C. rubella*) showed a $K_A/K_S > 1$ in any region (Figure S2), suggesting that *GEX3* has not evolved under positive selection. Rather, the K_A/K_S ratio is lower than 1, especially at the N-terminus of the protein, suggesting that *GEX3* evolved under purifying selection.

GEX3 Is Located at the Plasma Membrane

Several different protein localization algorithms predict that GEX3 has a signal peptide and is anchored in the plasma membrane. To test these predictions, a protein fusion construct (*Arabidopsis GEX3* cDNA fused to eGFP at the C-terminus, under the control of the *CaMV35S* promoter) was introduced into *Arabidopsis*. The sub-cellular localization of GEX3–eGFP was investigated using fluorescence microscopy in roots of 3–4-d-old seedlings. Figure 2 shows that the GFP signal was observed at the cell surface of elongated epidermal cells as well as in cells of the root tip. That GEX3–eGFP is at the plasma membrane of the root cells was corroborated by co-localization with the vital dye FM4-64 (Figure 2A, 2B, and 2C), which specifically stains phospholipids of the plasma membrane (Pagant et al., 2002). In the root tip, where the GFP signal was very bright, GEX3–eGFP was also perinuclear (Figure 2D and 2E), likely in the ER (Roselli et al., 2004). Plasma membrane proteins that are highly expressed are sometimes also found in the ER, because the constitutive exocytic pathway is overwhelmed (Beraud-Dufour and Balch, 2002; Roselli et al., 2004). High expression likely explains the perinuclear localization of GEX3–eGFP in the root tip, since perinuclear fluorescence was not observed in the root epidermal cells, where expression was weaker.

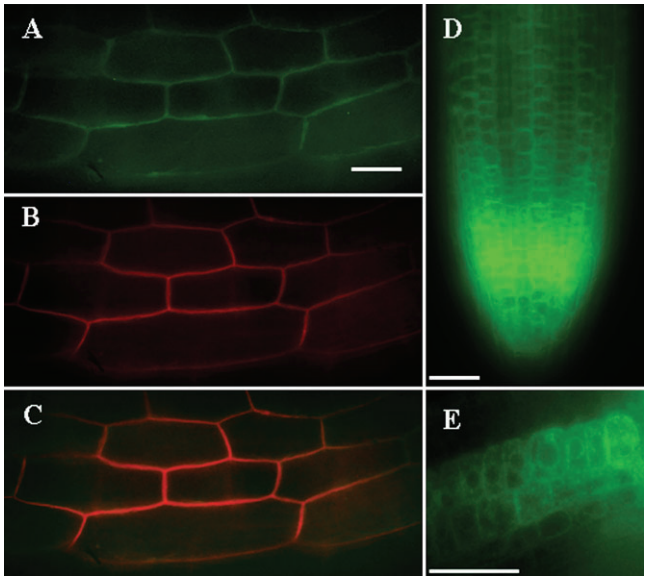

Figure 2. *Arabidopsis* GEX3 Is Plasma Membrane-Localized.
(A) Plasma membrane localization of GEX3–eGFP in root epidermal cells.
(B) FM4-64 dyes the lipids of the plasma membrane in root epidermal cells.
(C) Merged image of (A) and (B).
(D) GEX3–eGFP is localized at the plasma membrane and perinuclearly in the root tip cells of *Arabidopsis*.
(E) Close-up of (D). Bars = 20 μm.

GEX3 Is Expressed in the Male Gametophyte and in Mature Siliques

GEX3 is not represented on the Affymetrix gene chip (http://affymetrix.arabidopsis.info), so there are no microarray expression data for it. We therefore tested its expression in different *Arabidopsis* tissues by RT–PCR and found that it was expressed in pollen and in mature siliques (6–8 d after pollination DAP) (Figure 3A). To determine which cell types expressed *GEX3*, we first generated a GUS reporter line under the control of the *Arabidopsis GEX3* promoter (*ProGEX3:GUS*), but expression was weak/not discernable. Because tdTomato is a very

bright fluorescent protein (Shaner et al., 2004), we tested a construct in which the *GEX3* promoter was driving expression of tdTomato, and obtained six lines with expression in the sperm cells (example shown in Figure 3B, left panel). Expression in sperm cells was expected, since we found two *GEX3* ESTs from maize sperm cells (Engel et al., 2003), and there were *GEX3* ESTs from sperm cells of rice and plumbago. In three of these lines, we also observed weak expression of *ProGEX3:tdTomato* in the vegetative cell (example shown in Figure 3B, right panel). Because we found the same expression pattern in several reporter lines, and because *ProGEX3:tdTomato* was expressed in the vegetative cell of tobacco in a pollen bombardment assay (data not shown), we think that expression in the vegetative cell is detectable when the transgene is strongly expressed, and is not due to transgene location within the genome.

Overexpression and Down-Regulation of *GEX3* Using the *GEX2* Promoter

A common approach to uncover the function of a gene is to overexpress and/or down-regulate it, followed by phenotypic analysis. We were interested in determining whether plants deficient in GEX3 function would show defects in fertilization.

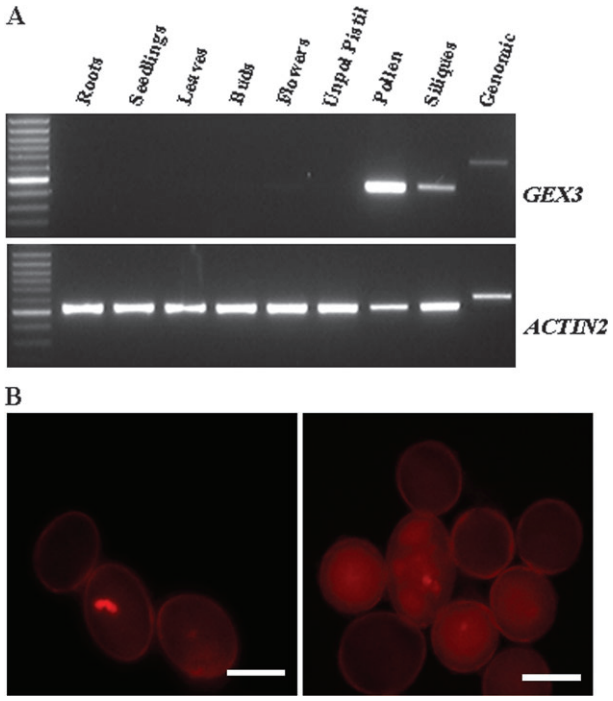

Figure 3. *Arabidopsis GEX3* Is Expressed in Pollen and Mature Siliques.
(A) RT–PCR of *GEX3* and *ACTIN2* in eight different tissues of *Arabidopsis*. Genomic DNA was loaded as a primer control.
(B) Expression of *ProGEX3:tdTomato* in the sperm (left and right panels) and vegetative cells of pollen (right panel). Bars = 20 μm.

Plants that are heterozygous for male gametophytic mutants that affect pollen tube growth will still show full seed set, because the 50% wild-type (WT) pollen will fertilize all available ovules (McCormick, 2004). However, plants that are heterozygous for male gametophytic mutants affecting the last steps of the fertilization process might show reduced seed set, if WT pollen tubes cannot access ovules that had already been reached by a mutant pollen tube, as was shown for the *hap2* mutant (von Besser et al., 2006). Plants that are heterozygous for female gametophytic mutants and that show a defect in fertilization carry 50% non-fertilized ovules or aborted seeds (Yadegari and Drews, 2004). To test whether a disruption in *GEX3* resulted in reduced seed set, we first analyzed two SALK T-DNA insertion lines that reportedly targeted the last exon of *GEX3*. For one of the lines (S_ 084796), the T-DNA insertion was mapped, after PCR amplification and sequencing, to the 3′UTR of *GEX3* and homozygous plants for this T-DNA were obtained. Because these plants did not show a reduced seed set phenotype, we concluded that this T-DNA insertion did not interfere with the function of GEX*3*. For the second T-DNA insertion line (S_002030), we could not amplify the insertion by PCR in either orientation, using primers flanking the location of the T-DNA, according to TAIR.

Therefore, to uncover a role for GEX3 during fertilization, we used the promoter of *GEX2* to generate transgenic lines that overexpressed or down-regulated *GEX3* in the sperm cells (Figure 4A). *GEX2* from *Arabidopsis* was shown to be transcriptionally active in the generative cell and its progeny, the sperm cells (Engel et al., 2005). We screened for a reduced seed set phenotype in the T1 generation of the transgenic lines. For each construct, we identified 10 primary transformant (T1) plants that showed reduced seed set (compare Figure 4E with 4F and 4G), which ranged from 30 to 50% reduced seed set. Two lines from each construct that showed a strong phenotype (~50% reduced seed set) were characterized further in the T2 and T3 generations. These antisense lines were named AS4 and AS27 and the overexpression lines were named S6 and S12 (Figure S3).

Down-Regulation of *GEX3* Disrupts Micropylar Pollen Tube Guidance

The antisense transgenic lines showed approximately 25–30% pollen abortion. However, the viable pollen was tricellular (Figure 4C), indicating that pollen development was not delayed and that the two pollen mitoses (PMI and PMII) occurred normally (compare Figure 4B and 4C). When we selected the T2 generation of a self-cross using the linked Basta resistance gene, we found a segregation ratio of 1:1 Resistant to Sensitive seedlings (349:340), indicating aberrant transmission through one or both of the parents. These transgenic lines had 50% non-fertilized ovules. In order to determine which gametophyte was responsible for the phenotype, we carried out reciprocal crosses of the antisense lines with WT plants and then scored siliques for reduced seed set. Figure 5A shows that when the transgenic lines were used as females in crosses

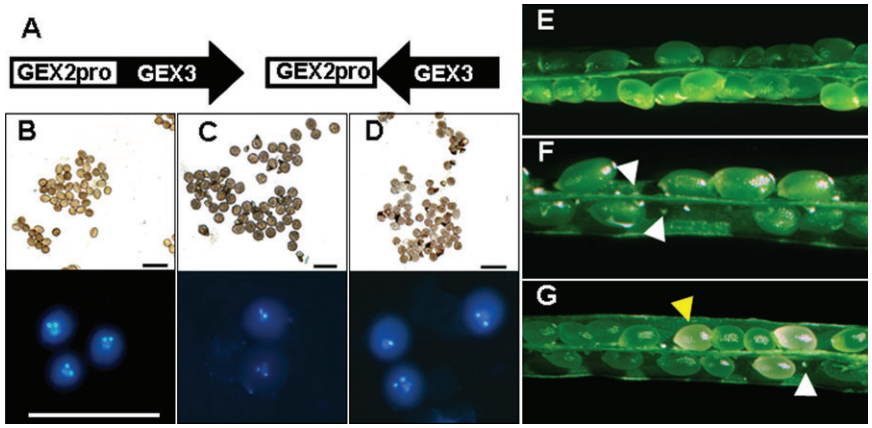

Figure 4. *GEX3* Antisense and Sense Constructs and Phenotypes of Transgenic Lines.
(A) Diagram of constructs used to down-regulate (antisense, AS) or overexpress (sense, S) *GEX3* using the *GEX2* promoter.
(B) WT pollen. Bright field image (top panel), stained with DAPI (bottom panel).
(C, D) Bright field (top panels) and DAPI staining (bottom panels) of pollen from the *GEX3* AS27 and S12 lines, respectively.
(E) Mature green silique of WT (6–8 d after pollination, DAP) with full seed set.
(F) Mature silique of *GEX3* AS27 line (6–8 DAP), showing non-fertilized ovules (white arrows).
(G) Mature silique of *GEX3* S12 line (6–8 DAP), showing non-fertilized ovules (white arrows) and white seeds (yellow arrows).
Bar = 50 μm.

with WT pollen, ~50% of the ovules were non-fertilized. When we used the transgenic lines as pollen donors in crosses with WT plants, only ~7% of the ovules were non-fertilized. The small percentage of non-fertilized ovules in these crosses was likely due to a slight problem with anther dehiscence in the AS27 transgenic line. These data indicate that the female gametophyte was mostly responsible for the reduced seed set found in the *GEX3* antisense lines.

To determine which step of the fertilization process was affected in these transgenic lines, we collected self-crossed pistils 1–2 d after pollination and stained them with aniline blue to visualize *in vivo* pollen tube growth. About 50% of the ovules of each pistil were not fertilized. Of these unfertilized ovules, 62% (n = 100) had a pollen tube turning towards the funiculus but not reaching the micropyle, while the other 38% had no pollen tube. Figure 6A shows a fertilized ovule with a pollen tube in the micropyle and a non-fertilized ovule with a pollen tube on the funiculus that did not reach the micropyle. Figure 6B and 6C show two other non-fertilized ovules that had pollen tubes turning towards the funiculus. We observed a similar phenotype in crosses carried out using the transgenic lines as female with a *ProLAT52:GUS* reporter line as male. Figure 6G and 6H show a WT ovule targeted by a blue pollen tube and a mutant ovule in which a pollen tube turned towards the funiculus but failed to enter the micropyle. To confirm that this phenotype was not due to immature or defective embryo sacs (Drews et al., 1998), we used differential interference contrast (DIC) imaging (Figure S4A) to show that the female gametophytes had the expected numbers and normal positioning of cells.

To correlate the phenotype observed in the transgenic lines with the expression of *GEX3*, we used real-time PCR to quantify the *GEX3* transcript levels, with RNA isolated from anthers and un-pollinated pistils. Figure 5B shows that mRNA levels in the anthers and un-pollinated pistils of the *GEX3* antisense lines were ~50% lower than in WT. We do not have an antibody for GEX3 to quantify protein levels, but the reduction in the mRNA level is expected to reduce the protein level.

Over-Expression of *GEX3* Disrupts Pollen Tube Guidance and Sometimes Yields Non-Viable Embryos

The *GEX3* overexpression lines had about 20% aborted pollen, although the viable pollen was at the tricellular stage (Figure 4D). As with the *GEX3* antisense lines, when the *GEX3* overexpression lines were plated on Basta, the segregation ratio from the T2 generation of a self-cross was 1:1 Resistant to Sensitive seedlings (368: 348), indicating that the *GEX3* overexpression lines also exhibited aberrant transmission through one or both parents. The *GEX3* overexpression lines showed ~50% reduced seed set. Within the 50%, ~40% were non-fertilized ovules while the remaining 10% were white seeds (Figure 4G, white and yellow arrows, respectively). We carried out reciprocal crosses with wild-type plants, and then scored siliques for reduced seed set. Figure 5A shows that when the transgenic lines were females, there were ~45% non-fertilized ovules, but when the transgenic lines were male, there were only a few non-fertilized ovules, as is occasionally seen even in self-crosses of WT. These data indicated that the reduced seed set observed in the *GEX3* overexpressing lines was due to a defect in the female gametophyte.

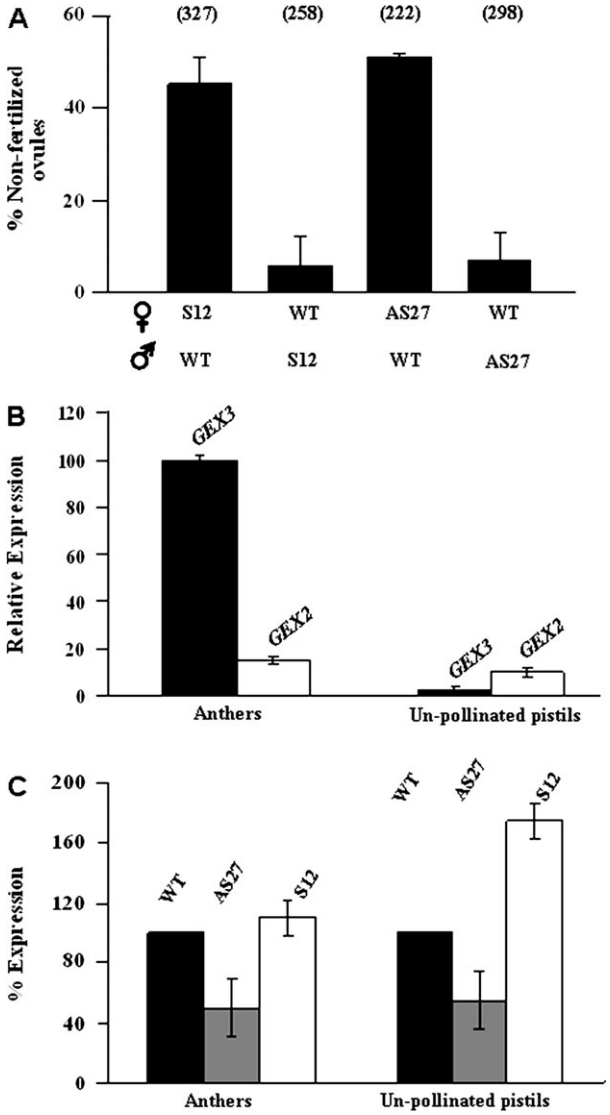

Figure 5. Transmission Genetics and mRNA Quantification in *GEX3* Antisense (AS) and Sense (S) Lines.

(A) Percentage of non-fertilized ovules in mature green siliques (6–8 DAP) after performing reciprocal crosses with WT plants. Numbers in parentheses indicate number of seeds counted from each cross.

(B) Relative expression of *GEX3* and *GEX2* mRNA levels in anthers and un-pollinated pistils of WT plants. The expression level of *GEX3* in anthers was set at 100%, and the other values were normalized using the $2^{-\Delta\Delta t}$ method (Livak and Schmittgen, 2001). Absolute values were generated with real-time PCR using cDNA from anthers (500 ng total RNA) and un-pollinated pistils (4 μg total RNA).

(C) *GEX3* mRNA levels measured using real-time PCR in anthers (500 ng total RNA) and un-pollinated pistils (4 μg total RNA) of WT, AS27, and S12 lines.

When we visualized *in vivo* pollen tube growth within pistils 1–2 d after pollination, we observed that 50% of the ovules of each pistil were not fertilized; with 69% (n = 97) of these ovules showing no nearby pollen tubes and the remaining 31% having a pollen tube reaching the micropyle. Figure 6D shows two ovules with pollen tubes reaching the micropyle; however, only one of the ovules was fertilized. Figure 6E and 6F show two other non-fertilized ovules with pollen tubes reaching the micropyle. We observed a similar phenotype when we carried out crosses using these transgenic lines as female and a *ProLAT52:GUS* reporter line as male. Figure 6I shows a WT ovule and a mutant ovule, both with pollen tubes in the micropyle, but only the WT ovule was fertilized. As we found for the antisense lines, the constitution and positioning of cells in embryo sacs of the overexpression lines were normal (Figure S4B).

To correlate the phenotype observed in the overexpressing lines with the expression of *GEX3*, we used real-time PCR to quantify the level of *GEX3* transcript. Figure 5B shows that in the un-pollinated pistils of the *GEX3* overexpression lines, *GEX3* mRNA levels were nearly twice (175%) that in WT pistils; however, in the anthers, the mRNA level was only slightly increased (110%) from that in WT anthers. The increased mRNA level in un-pollinated pistils is predicted to result in more GEX3 protein.

GEX3 and *GEX2* Are Also Expressed in the Female Gametophyte

In the *GEX3* antisense or overexpression lines, in which the *GEX2* promoter was used to drive gene expression, the reduced seed set phenotype was caused by a defect in the female gametophyte. These data indicated that both genes, *GEX3* and *GEX2*, must be expressed within the female gametophyte. To confirm this, we tested *GEX3* and *GEX2* expression in WT un-pollinated pistils using real-time PCR, and were able to detect expression of both genes (Figure 5B). For both genes, the levels of expression in WT un-pollinated pistils were much lower than in WT anthers. Moreover, the expression of *GEX3* was approximately four times lower than that of *GEX2* in un-pollinated pistils, but *GEX3* expression was approximately 10 times higher than that of *GEX2* in anthers. A previously used reporter construct that showed expression of *GEX2* in sperm cells, *ProGEX2:eGFP* (Engel et al., 2005), failed to show expression in the female gametophyte. Therefore, we made a new construct, *ProGEX2:tdTomato*, confirmed expression in sperm cells (data not shown) and screened those lines for expression in the female gametophyte. We also examined the *ProGEX3:tdTomato* lines (Figure 3) for expression in the female gametophyte. Figure 7B and 7C, respectively, show expression of *ProGEX3:tdTomato* and *ProGEX2:tdTomato* in the egg cell within the female gametophyte. The fluorescence signal was very weak in both reporter lines, which is consistent with the low mRNA levels for *GEX3* and *GEX2* in un-pollinated pistils of WT plants (Figure 5B). Expression *ProGEX3:tdTomato* in the egg cell was detectable in reporter lines that expressed *GEX3* in

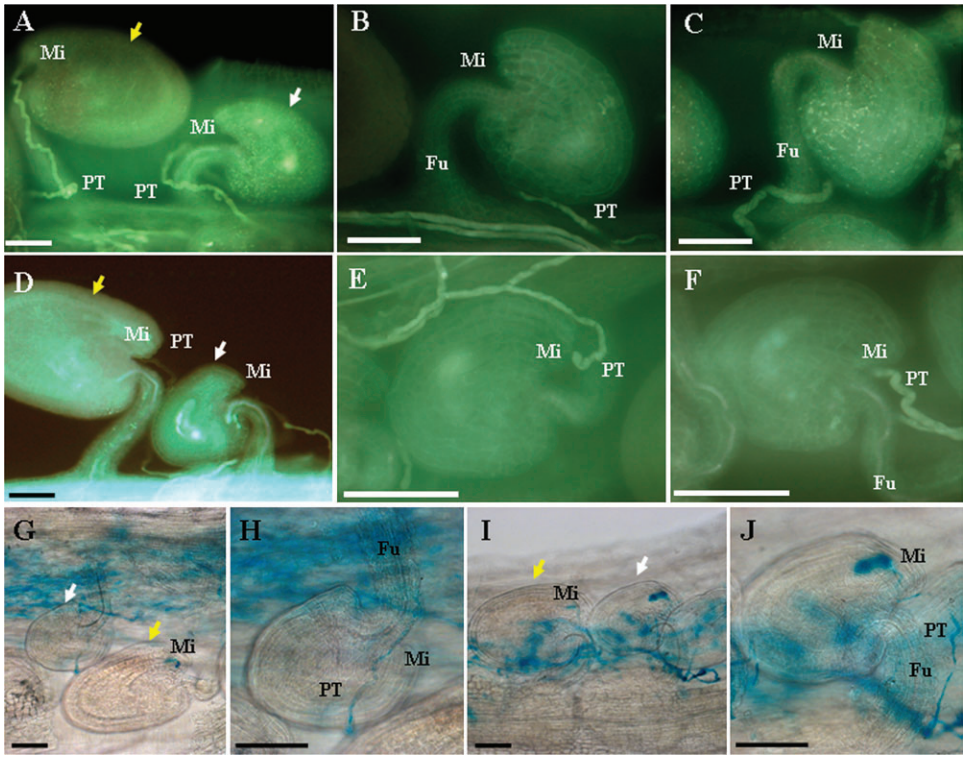

Figure 6. *In Vivo* Pollen Tube Growth in *GEX3* Antisense and Sense Lines.
(A) Fertilized and non-fertilized ovule, yellow and white arrow respectively, in a *GEX3* antisense line.
(B, C) Two non-fertilized ovules of an At *GEX3* antisense line, with pollen tubes that did not reach the micropyle.
(D) Fertilized and non-fertilized ovule, yellow and white arrow respectively, in a *GEX3* sense line.
(E, F) Two non-fertilized ovules of a *GEX3* sense line with a pollen tube at the micropyle.
(G) 1DAP pistil from a cross using a *GEX3* antisense line as female with *ProLAT52:GUS* line as a male. A WT ovule (yellow arrow) was fertilized (blue pollen tube at the micropyle) and the mutant ovule (white arrow) was not fertilized.
(H) Close-up of (G). The pollen tube missed the micropyle and continued growing on the mutant ovule.
(I) 1DAP pistil from a cross of a *GEX3* sense line as female and a *ProLAT52:GUS* line as male. A WT ovule (yellow arrow) was fertilized (blue pollen tube at the micropyle) and the mutant ovule (white ovule) was not fertilized.
(J) Close-up of (I). The pollen tube reached the micropyle and burst there, although fertilization failed. Bar = 20 μm. Mi, micropyle; PT, pollen tube; Fu, funiculus.

both the vegetative and sperm cells, and in lines showing expression only in the sperm cells. *GEX3* was not represented in the the ESTs sequenced from a maize egg cell cDNA library (Yang et al., 2006), but the GEX3 transcript could be detected with RT–PCR (Figure S5), using cDNA from maize egg cells (Yang et al., 2006).

White Seeds in *GEX3* Over-Expression Lines Carry Non-Viable Embryos

To determine whether the white seeds observed in the siliques of the overexpression lines carried viable embryos, we dissected and cleared the seeds from siliques at different developmental stages and visualized them using DIC microscopy. Figure 8A–8C show WT embryos at the triangular, late heart and torpedo stages, respectively. Figure 8D–8F show the morphology typical of a non-viable embryo from a white seed. Non-viable embryos already had an abnormal shape at the triangular stage and arrested development at the heart stage. Non-viable embryos had an enlarged shoot apical meristem (SAM), as shown in Figure 8C and 8D. Figure 8G shows a WT embryo at the cotyledon stage and Figure 8H shows a non-viable embryo from the same mature silique (8–10 DAF).

DISCUSSION

GEX3 Is Expressed in the Male Gametophyte and in the Egg Cell

We were interested in uncovering a possible function for GEX3 in fertilization. RT–PCR of mRNA from different tissues of *Arabidopsis* showed that *GEX3* was expressed in pollen and in

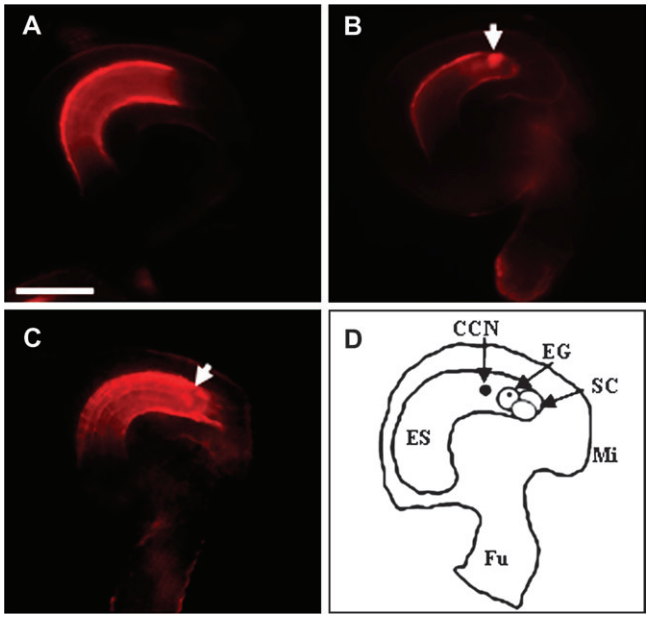

Figure 7. *GEX3* and *GEX2* Are Expressed in the Egg Cell.
Cellular expression patterns of *GEX3* and *GEX2* within the female gametophyte were detected in the reporter lines *ProGEX2:tdTomato* and *ProGEX3:tdTomato*.
(A) WT ovule.
(B) *GEX3* expression in the egg cell (arrow).
(C) *GEX2* expression in the egg cell (arrow).
(D) Diagram of a female gametophyte. Bar = 50 μm. Mi, micropyle; Fu, funiculus; ES, embryo sac; SC, synergid cells; EG, egg cell; CCN, central cell nucleus.

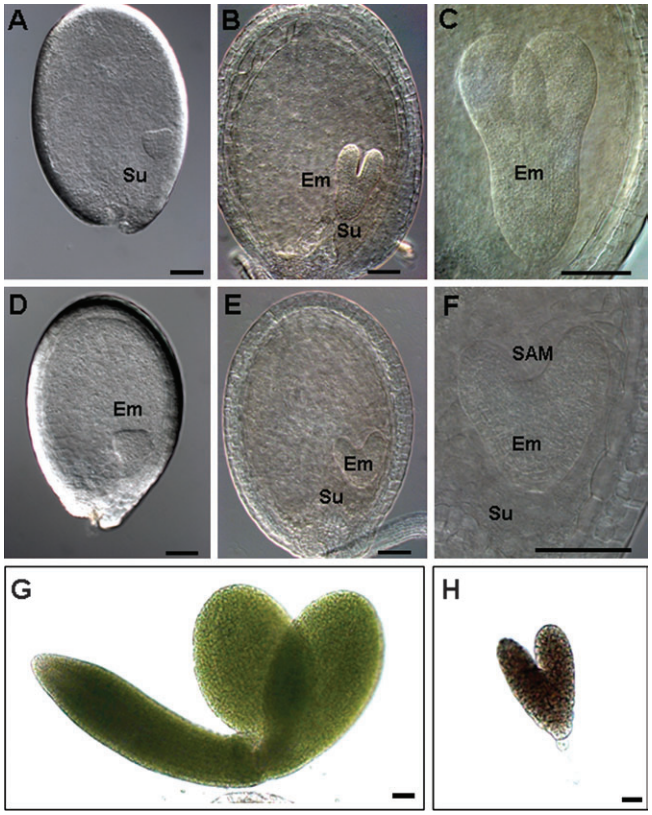

Figure 8. Non-Viable Embryos in White Seeds of *GEX3* Sense Lines.
(A–C) WT embryo development at the triangular, late heart and torpedo stages, respectively.
(D–E) Non-viable embryos in the white seeds of the *GEX3* sense lines, at the triangular and heart stage, respectively.
(F) Close-up of (E).
(G) WT embryo at the cotyledon stage.
(H) Non-viable embryo, arrested at the heart stage, from the same silique (10 DAP) as the WT embryo in (G). Bar = 50 μm.
Em, embryo; Su, suspensor; SAM, shoot apical meristem.

mature green siliques (Figure 3A), and analyses of reporter lines carrying *ProGEX3:tdTomato* showed that *GEX3* was strongly expressed in the sperm cells and sometimes weakly expressed in the vegetative cell (Figure 3B). To down-regulate and overexpress *GEX3*, we chose to use the previously characterized *Arabidopsis GEX2* promoter, which was active in generative and sperm cells (Engel et al., 2005). Both antisense and overexpression transgenic lines for *GEX3* had reduced seed set (Figure 4F and 4G), but, surprisingly, the phenotype, in both cases, was caused by a defect on the female side (Figure 5A). These results indicated that both genes, *GEX2* and *GEX3*, must be expressed in the female gametophyte, even though RT–PCR from pistils (Engel et al., 2005) and in Figure 3A failed to show it. We therefore performed real-time PCR using un-pollinated pistils and were able to detect expression of both genes; *GEX2* expression was approximately four times that of *GEX3* (Figure 5B). Furthermore, expression in the egg cell was detectable in transgenic lines expressing either *ProGEX2:tdTomato* or *ProGEX3:tdTomato* (Figure 7B and 7C), although the signal was weak. The expression of *GEX2* and *GEX3* in the egg cell fits with the reduced seed set phenotype observed in the transgenic lines.

Down-Regulation of *GEX3* Blocks Fertilization Due to a Defect in Micropylar Pollen Tube Guidance

The *GEX3* antisense lines had a defect in fertilization on the female side (Figure 5A), although the development of the female gametophyte was not affected (Figure S4A). Fertilization was blocked in these transgenic lines due to a defect in micropylar pollen tube guidance (Figure 6A–6C) and mRNA levels of *GEX3* were reduced to 50% in un-pollinated pistils of these antisense lines (Figure 5C), likely generating a reduction in the protein level.

We could only detect expression of *GEX3* in the egg cell within the female gametophyte (Figure 7B), so conclude that the presence of GEX3 in the egg cell is essential for micropylar pollen tube guidance in the female gametophyte.

Although real-time PCR showed reduced *GEX3* mRNA levels in anthers of the antisense lines (Figure 5C), this reduction was not

responsible for the reduced seed set phenotype. We observed 25% pollen abortion in these transgenic lines (Figure 4C), which could have been caused by *GEX3* down-regulation. However, a recent report (Xing and Zachgo, 2007) stated that some pollen abortion is observed in ~10% of transgenic plants, regardless of the construct type used. This phenomenon reportedly can affect more than 20% of transgenic plants in which RNAi technology is applied, wherein up to 20–50% non-viable pollen could be observed (Xing and Zachgo, 2007).

Reciprocal crosses (Figure 5A) performed with WT plants and crosses using the reporter line *ProLAT52:GUS* as pollen donor (Figure 6G and 6H) demonstrated that the 50% non-fertilized ovules phenotype in the antisense lines was due to a problem on the female side. In addition, the 1:1 ($Basta^R$:$Basta^S$) ratio in selfed progeny of a plant heterozygous for the antisense construct indicated that only one parent was responsible for the reduced seed set. We therefore think that pollen in which *GEX3* was down-regulated were not impaired in any step of the fertilization process.

To drive the antisense construct, we used the *Arabidopsis GEX2* promoter, which we thought was transcriptionally active only in the generative and sperm cells of the male gametophyte (Engel et al., 2005). We know that *GEX3* is expressed in both the sperm and the vegetative cell (Figure 2B and 2C). However, we cannot tell whether the *GEX3* antisense construct was only down-regulating the expression of *GEX3* in the sperm cells or was also affecting the expression in the vegetative cell because, as von Besser et al. (2006) suggested, there might be communication between the sperm cells and the vegetative cell during pollen tube growth. In the antisense lines, the lack of a phenotype on the male side could be explained by two hypotheses: (1) GEX3 in sperm cells is not essential for fertilization or (2) the absence of GEX3 is compensated by other molecules (on the assumption that *GEX3* expression was substantially down-regulated in the sperm cells of pollen carrying the antisense construct). *GEX3* expression in the male gametophyte is four times higher than that of *GEX2*. Therefore, it is possible that the *GEX3* antisense construct was only partially down-regulating *GEX3* and that the remaining *GEX3* was sufficient for fertilization. Therefore, we cannot rule out the possibility that GEX3 also plays a role in pollen tube guidance in the male gametophyte.

Over-Expression of *GEX3* Affects Pollen Tube Guidance and Generates a Few Non-Viable Embryos

Transgenic lines overexpressing *GEX3* also had reduced seed set, which was due to a problem on the female side (Figure 5A), although the development of the female gametophyte was not affected (Figure S4B). Unlike the antisense lines, the overexpression lines had a mixed phenotype of 40% non-fertilized ovules and 10% white seeds carrying non-viable embryos (Figure 3G). Most of the overexpressing ovules did not attract pollen tubes at all, although ~30% of them had pollen tubes in the micropyle (Figure 6D–6F). Among the ovules that attracted pollen tubes, 20% were not fertilized and the remaining 10% formed non-viable embryos (Figure 8D–8F). We think that, sometimes, pollen tube discharge or sperm cell release occurred in the overexpressing ovules that did attract pollen, because pollen tube discharge was evident (Figure 6J), even though fertilization was blocked. The presence of 10% non-viable embryos also supports this argument. When we performed real-time PCR in un-pollinated pistils of the overexpressing lines, we detected nearly twice (175%) as much *GEX3* mRNA as in WT (Figure 5C), which probably resulted in increased protein levels. The ability of some overexpressing ovules to attract pollen tubes or to be fertilized could be related to differences in transcript levels. In any case, the non-viable embryos indicate that GEX3 must also play a role during early embryogenesis, and is consistent with the expression seen in mature green siliques (Figure 3A). Unfortunately, because of autofluorescence in the tdTomato channel, we were unable to detect expression of *GEX3* during early embryogenesis in the tdTomato reporter line.

The overexpression lines also showed ~20% pollen abortion (Figure 4D), presumably due to previously stated reasons (Xing and Zachgo, 2007). The anthers of the overexpressing lines did not show up-regulation of the *GEX3* mRNA levels (Figure 5C). This is probably because the level of *GEX2* expression in this tissue is 10 times lower than *GEX3* (Figure 5B); therefore, the promoter used in the transgene did not increase *GEX3* mRNA levels.

GEX3, a Conserved Plant Protein Located at the Plasma Membrane, Contains PQQ Domains and Evolved under Purifying Selection

In root epidermal cells, GEX3–eGFP was co-localized at the plasma membrane with the dye FM4-64 (Figure 2C). We think that the ER localization in the root tip (Figure 2D and 2E) was due to the high expression of the *Pro35S:GEX3–eGFP* construct in these cells. *GEX3* was found in 13 plant species, including monocots and dicots (Figure1B, Figure S2) and in the lycophyte *Selaginella moellendorffii* (not shown, http://selaginella.genomics.purdue.edu), but not in the moss *Physcomitrella patens* (Rensing et al., 2007), even though both mosses and lycophytes have sperm and egg cells. Comparisons of the open reading frames of *GEX3* from four species that are phylogenetically closely related showed that *GEX3* has evolved under purifying selection, especially at its N-terminus (Figure S1). Proteins or domains within a protein that evolve under negative or purifying selection do not accept changes in their amino acid sequence, and, accordingly, show high levels of sequence conservation over long evolutionary times (Dias et al., 2003; Stewart et al., 2008). The purifying selection detected for *GEX3* therefore suggests that GEX3 requires particular amino acids for its function (Rohmer et al., 2004).

Depending on the species, GEX3 has three to five b-propeller repeats (PQQ domains) at the N-terminus, which is predicted to be extracellular (Figure 1B). Quinoproteins are very abundant in bacteria, as dehydrogenase enzymes that use pyrrolo–

quinoline quinone as a redox cofactor. Quinoproteins have also been found in eukaryotes (Kasahara and Kato, 2003; Felton and Anthony, 2005). In plants, there are proteins annotated as quinoproteins in Rice, Cleome, Plumbago, and *Arabidopsis* (e.g. quinoprotein alcohol dehydrogenase-like, At5g11560 and ire-1 homolog, At5g24360). PQQ was reported in pistils and pollen grains of *Lilium longiflorum*, and some other higher plants (Xiong et al., 1990), and PQQ stimulated *in vitro* pollen germination in lily, in a concentration-dependent manner (Xiong et al., 1988). These reports, together with the phenotypes we observed (Figures 2, 4, and 6) suggest that PQQ might be a new extracellular cue that is perceived by GEX3.

Pollen Tube Guidance Requires Multiple Signals

The synergids play an important role in pollen tube guidance (Higashiyama et al., 2001; Shimizu and Okada, 2000). The central cell also plays a role in micropylar pollen tube guidance, as deduced by the phenotypes of mutations in the *Arabidopsis* gene *CCG*, which encodes a nuclear protein which may act as a transcription factor (Chen et al., 2007). Here, we showed that GEX3, a plasma membrane-localized protein expressed in the egg cell in *Arabidopsis*, plays a role in micropylar pollen tube guidance. This role for the egg in pollen tube guidance is similar to that shown for the mutant *eostre*, which has two functional egg cells and also showed a defect in pollen tube attraction (Pagnussat et al., 2007). At least one sperm-specific protein, HAP2/GCS1 (von Besser et al., 2006), plays a role in pollen tube guidance. Together, these findings suggest that pollen tube guidance requires multiple signals from different cells of the female and male gametophyte, that it is not only the synergid cells and the pollen tube which are important. Whether PQQ is a new signal involved in pollen tube guidance that acts through GEX3 on the plasma membrane of the egg cell remains to be elucidated. The identification of the signaling proteins from each of these cells will be necessary to fully understand pollen tube guidance.

METHODS

Plant Materials and Growth Conditions

Arabidopsis plants were grown in greenhouse conditions in a 4:1:1 mix of Fafard 4P:perlite:vermiculite under an 18-h light/6-h dark cycle at 21°C. The Col-0 ecotype was used for stable transformation (Clough and Bent, 1998). T1 transgenic plants were sown in soil, sprayed twice (approximately 6 and 8 d after germination) with a 1:400 dilution (final concentration 0.014% w/v) of Basta (Finale, Farnam Companies, Inc.) and left for another 7 d before scoring for resistance. Approximately 30 resistant plants were transferred to individual pots, genotyped, and phenotyped.

Generation of Transgenic Lines

Overexpressing or down-regulating lines of *Arabidopsis GEX3* were generated using the Gateway system (Invitrogen). We used the destination vector pB7WG2 from VIB-Ghent University (Karimi et al., 2002), in which the CaMV35S promoter (Pro35S) was removed using the enzymes *Sac*I and *Spe*I and replaced with the 1.7-Kb *Arabidopsis GEX2* promoter (Engel et al., 2005) using the same enzymes, to generate pB7WG2*–*Pro GEX2*. The full-length *Arabidopsis GEX3* open reading frame was amplified from pollen cDNA using primers F, 5′-TTGGATCCCTGCGACAATGGTGGCTTTTAGATTCG-3′ and R, 5′-TTGAATTCCCCTTAATTAGTCGTCAAAGATATGC-3′ for the sense construct, and F, 5′-TTGAATTCCTGCGACAATGGTGGCTTTTAGATTCG-3′ and R, 5′-TTGGATCCCCCTTAATTAGTCGTCAAAGATATGC-3′ for the antisense construct. The pENTR 3C vector (Invitrogen) was digested with *Eco*RI and *Bam*HI, then the *GEX3* open reading frame was inserted into it, in the sense or antisense orientation, and confirmed by sequencing. Sense and antisense *GEX3* cDNAs were transferred to the modified pB7WG2*–*ProGEX2* vector by an LR reaction using LR Clonase II (Invitrogen) to generate the *ProGEX2:GEX3* and *ProGEX2:GEX3AS* constructs.

For subcellular localization, the *GEX3* open reading frame without the stop codon was amplified from pollen cDNA, using primers F, 5′-CACCATGGTGGCTTTTAGATTCGTTTATATC-3′ and R, 5′-AGTCGTCAAAGATATGCTTCAAATATATG-3′ and cloned into the pENTR/D Topo vector (Invitrogen). After sequencing, the *GEX3* open reading frame was transferred to the destination vector pB7FWG2 (Karimi et al., 2002) by an LR reaction, to generate the *Pro35S:GEX3–eGFP* construct.

The reporter lines for *GEX2* and *GEX3* were generated using the pMCD163 plasmid (Curtis and Grossniklaus, 2003), which was digested with *Sac*I and *Spe*I to release the GUS reporter gene, and then the tdTomato gene (Shaner et al., 2004) was inserted as a *Sac*I–*Spe*I fragment, to generate pMDC–tdTomato. The promoter of *Arabidopsis GEX2* (1886 bp) was amplified using the primers F, 5′- CACCGGTCTGATTTCTTACATCGGATGG and R, 5′-CAGATGGGTATATTAACCCTTCACAACAAG-3′. The promoter of *Arabidopsis GEX3* (1498 bp) was amplified using the primers F, 5′-CACCTACACACCTGCGTTTCTAGCAGCTGC-3′ and R, 5′-TGTCGCAGATTTTTGCAGAGAGGTG-3′. Both promoters were cloned into pENTR D/Topo (Invitrogen) and their sequences verified. The pENTR clones were digested with *Ava*I for Pro*GEX2* and *Eco*RV for Pro*GEX3* and then the promoters were transferred into pMDC–tdTomato by an LR reaction using LR Clonase II (Invitrogen) to generate *ProGEX2:tdTomato* and *ProGEX3:tdTomato*.

ESTs, Protein Sequence and Positive Selection Analyses

The sperm cell database (Engel et al., 2003) as well as other maize cDNA libraries were searched using the TIGR database (http://maize.tigr.org). Two overlapping sperm-expressed ESTs for *GEX3* were found: Zmsp8743 (Genbank #CF602491) and Zmsp8903 (Genbank #CF602594). The full-length genomic sequence of maize *GEX3* (MAGI4_36086:1560) was obtained from the MAGI database (www.plantgenomics.iastate.edu/maize). Orthologs of maize *GEX3* were found in other species using BLAST (www.ncbi.nlm.nih.gov/BLAST): *Oryza sativa*

sperm cells EST (Genbank # NP_001043519), *Plumbago zeylanica* sperm cells EST (Genbank # CB818224), *Cirsium arvense* mixed reproductive tissues EST (Genbank # DV175248), and genomic DNA sequences of *Cleome spinosa* (ABD96909.1), *Populus trichocarpa* (fgenesh4_pg.C_LG_IV000891, http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), *Eucalyptus gunnii* (CT987017.1), *Vitis vinifera* (EC937052.1), and *Sorghum bicolor* (SAMIv2_37783, www.plantgenomics.iastate.edu/maize).

The structure and subcellular localization of *Arabidopsis* GEX3 was predicted with PSORT (http://psort.nibb.ac.jp/), TargetP1.1 (www.cbs.dtu.dk/services/TargetP/), TMHMM (www.cbs.dtu.dk/services/TMHMM), TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), and Phobious (http://phobius.cgb.ki.se/). Predictions of protein domains were made using SMART (http://smart.embl-heidelberg.de).

Two primers that were complementary to the *A. thaliana*, *Z. mays*, and *O. sativa* sequences were designed at the 5′ and 3′ ends of *GEX3*:primer F, 5′- GGCAGAGTCTACGCTTGCTCAG-3′ and primer R, 5′- ATATATGCTTCTTCTACTGCTTGCA-3′. Genomic sequences of *GEX3* from *Arabidopsis lyrata*, *Capsella rubella*, and *Cardamine hirsuta* were amplified (2271 bp) using Phusion Taq polymerase (New England Biolab) and sequenced. We used dnasp software (www.ub.es/dnasp/) to perform sliding window analyses between the *GEX3* sequences from *A. thaliana*, *A. lyrata*, *C. rubella*, and *C. hirsuta*. We used one-tenth of the analyzed sequence as the window length (170 bp) and half of the window length (85 bp) as the step size, as in Escobar-Restrepo et al. (2007).

Phenotypic Analysis

Seed set was analyzed 8–10 d after pollination in siliques from T1 and T2 transgenic lines and in Col-0. Siliques were placed on double-sided tape and transversely dissected under a stereoscope to score seed set. Images of siliques were taken with a Nikon SMZ800 stereoscope. For reciprocal crosses with Col-0, flowers were emasculated at night and crosses were carried out 18–20 h later. Siliques were analyzed for reduced seed set after 8–10 d. Pollen tube growth in pistils was analyzed using aniline-blue staining, in pistils 1–2 d after pollination, as described (Mori et al., 2006). GUS expression in pistils pollinated with pollen from *ProLAT52:GUS* plants were analyzed, as described (Johnson et al., 2004). Ovules and seeds were dissected and cleared overnight in Hoyers solution, as described (Pagnussat et al., 2005). Un-fertilized ovules were fixed in FAA (3.5% formaldehyde, 5% acetic acid and 50% ethanol in water) for 90 min, and then washed with water for 5 min before clearing. Images were taken with a Zeiss Axiophot microscope (Zeiss, Jena, Germany) with bright-field, epifuorescence or DIC optics using an AxioCamRM camera and AxioVision 4.3 software. All images were processed using Adobe Photoshop 7.0 (Adobe).

RT–PCR

RNA was extracted from different tissues of Col-0 using an RNeasy plant miniprep kit (Qiagen) with on-column DNAse treatment. cDNA was synthesized using 20-mer oligo d(T) and SuperScript™ III Reverse Transcriptase (Invitrogen). RT–PCR (30 cycles) with *Arabidopsis* tissues was performed using 1 μg of total RNA. *GEX3* primers for *Arabidopsis* were: F, 5′-TGGACGAGAGGATTGCTCTTGCG-3′ and R, 5′-CCAAGACTATAGGTCGACGAGAGTTT-3′, *ACTIN* primers: F, 5′- GTTAGCAACTGGGATGATATGG-3′ and R, 5′-CAGCACCAATCGTGATGACT TGCCC-3′. RT–PCR (35 cycles) with cDNA from maize egg cells (Yang et al., 2006) was performed. *GEX3* primers for maize were: F, 5′- CCGTCAGTGGCAGCTACTCG-3′ and R, 5′-GGAGAAATCGAGTAGAGGGCCC-3′. PCR products were visualized on an agarose gel containing ethidium bromide. Real-time PCR primer pairs were designed using Primer Express software (Applied Biosystems). *IPP2* primers: F, 5′- GTATGAGTTGCTTCTCCAGCAAAG-3′ and R, 5′- GAGGATGGCTGCAACAAGTGT-3′; *GEX3* primers: F, 5′- ATTGATTCTTGCGAAGGAAGCA-3′ and R, 5′- TAAGTGCCACGGAGAGATAACG-3′, and *GEX2* primers: F, 5′- GGATCAAGTCACCAAGCTGCTACT-3′ and R, 5′- TCACTAGGTTGCCTGAAGCTCTT-3′. Amplification was performed with a MyIQ Real-Time PCR Detection System (BIO-RAD), with 4 μg and 500 ng of total RNA from un-pollinated pistils and anthers, respectively, using EvaGreen Dye (Biotium) and the following program: cycle 1, 3 min at 95°C; cycle 2, 40 times 10 s at 95°C followed by 30 s at 60°C; cycle 3, 1 min at 60°C; and cycle 4, 80 times 10 s at 60°C. The final volume of the PCR was 20 μl. The *IPP2* gene (*At3g02780*) was used as an internal control to normalize for variation in the amount of cDNA template. Each real-time PCR experiment contained four technical replicates. Two or three real-time PCRs were carried out with each biological replicate and two biological replicates (RNA from independently harvested tissues) were used. The relative gene expression levels were calculated using the $2^{-\Delta\Delta t}$ method (Livak and Schmittgen, 2001).

GEX3 Subcellular Localization and GEX3 Reporter Line Expression

T2 seedlings of *Arabidopsis* transgenic lines carrying the *Pro35S:GEX3–eGFP* construct were grown vertically on 1% agar plates without selection. Roots of 3–4-d-old seedlings were stained with FM4-64 dye, as described (Pagant et al., 2002), and then rinsed in water before observation. Pollen carrying the construct *ProGEX3:tdTomato* was placed on 2.5% gelatin-coated slides, hydrated with pollen germination buffer (with no sucrose) (Boavida and McCormick, 2007) and visualized. Ovules carrying the *Pro GEX3:tdTomato* construct were dissected and observed according to Steffen et al. (2007).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant* Online.

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