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Arabidopsis thaliana GEX1 has dual functions in gametophyte development and early embryogenesis

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SUMMARY

GEX1 is a plasma membrane protein that is conserved among plant species, and has previously been shown to be expressed in sperm cells and some sporophytic tissues. Here we show that *GEX1* is also expressed in the embryo sac before cellularization, in the egg cell after cellularization, in the zygote/embryo immediately after fertilization and in the pollen vegetative cell. We functionally characterize GEX1 in *Arabidopsis thaliana*, and show that it is a versatile protein that performs functions during male and female gametophyte development, and during early embryogenesis. *gex1-1/+* plants, which synthesize a truncated *GEX1* mRNA encoding a protein lacking the predicted cytoplasmic domain, but still targeted to the plasma membrane, had embryos that arrested before the pre-globular stage. *gex1-3/+* plants, carrying a null *GEX1* allele, had defects during male and female gametophyte development, and during early embryogenesis. Using an antisense *GEX1* transgenic line we demonstrate that the predicted GEX1 extracellular domain is sufficient and necessary for GEX1 function during the development of both gametophytes. The predicted cytoplasmic domain is necessary for correct early embryogenesis and mediates homodimer formation at the plasma membrane. We propose that dimerization of GEX1 in the zygote might be an upstream step in a signaling cascade regulating early embryogenesis.

Keywords: antisense, embryo sac, pollen, embryo lethal, plasma membrane protein, dimerization, BiFC.

INTRODUCTION

In flowering plants the mature male gametophyte (pollen grain) contains three cells: a large vegetative cell that 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 (Sundaresan and Alandete-Saez, 2010). The fertilization process begins when the pollen grain germinates on the female stigmatic cells, and ends when the two sperm cells fertilize the egg and the central cell to form the zygote and the primary endosperm cell, respectively.

Pollen tubes are guided through female tissues (Palanivelu *et al.*, 2003) until they turn towards an available ovule. The two synergid cells within the female gametophyte play important roles during pollen tube guidance, as shown in *Torenia fournieri* (Higashiyama *et al.*, 2001), *Zea mays* (Okuda *et al.*, 2009), and the Arabidopsis *mma1* and *maa3* mutants (Shimizu and Okada, 2000). The gametic cells also play a role in pollen tube guidance (von Besser *et al.*, 2006; Chen *et al.*, 2007; Alandete-Saez *et al.*, 2008). The pollen tube enters the micropyle through the filiform apparatus, then releases the two sperm cells within the degenerating synergid cytoplasm (Weterings and Russell, 2004). Pollen tube arrest after entering the micropyle was perturbed by mutations in several genes, including *FERONIA* (Escobar-Restrepo *et al.*, 2007), *ANXUR1/2* (Boisson-Dernier *et al.*, 2009), *LORELEI* (Capron *et al.*, 2008) and *AMC* (Boisson-Dernier *et al.*, 2008). In most cases, the two sperm cells will complete double fertilization, although single fertilization events may occur after the discharge of two sperm cells (Ron *et al.*, 2010) or after the discharge of only one sperm-like cell (Nowack *et al.*, 2006; Chen *et al.*, 2008; Frank and Johnson, 2009; Aw *et al.*, 2010). Upon double fertilization the development of the embryo and the endosperm is initiated.

Embryogenesis is a critical step in the life cycle of seed plants (Dumas and Rogowsky, 2008). The zygote undergoes its first unequal cell division to yield an apical cell, which will develop into the embryo proper, and a basal cell, which will form the suspensor (Guitton and Berger, 2005). The complex process of seed development can be conceptually divided into three phases: the first establishes the pattern of the embryo by rapid cell division; the second is characterized by cell expansion and accumulation of reserves; whereas during the third phase, the seed desiccates (Devic, 2008).

Here we show that Arabidopsis GEX1 is a versatile protein that functions during the development of the male and female gametophytes, and during early embryogenesis. *GEX1* is expressed in the embryo sac before cellularization, in the egg cell after cellularization, in the zygote/embryo immediately after fertilization and in the male gametophyte, in both the vegetative cell and in the sperm cells. We characterized two different *GEX1* mutant alleles: *gex1-1* and *gex1-3*. *gex1-1* plants had arrested embryos, whereas *gex1-3* plants had defects during male and female gametophyte development, and during early embryogenesis. We demonstrate that the predicted GEX1 extracellular domain is sufficient and necessary for GEX1 function during the development of male and female gametophytes. Finally, using bimolecular fluorescence complementation (BiFC), we show that GEX1 forms homodimers at the plasma membrane through its cytoplasmic domain, and that this cytoplasmic domain is necessary for early embryogenesis.

RESULTS

GEX1 in Arabidopsis (At5g55490) is the ortholog of a maize protein represented by one expressed sequence tag (EST) among 5000 ESTs from a sperm cell cDNA library (Engel *et al.*, 2003, 2005). GEX1 has a predicted topology of a signal peptide, a large extracellular domain (ECD) containing two coiled-coil domains, three transmembrane domains (TMDs) and a short cytoplasmic domain (CD), as shown in Figure 1(a). Consistent with this prediction, a GEX1-eGFP fusion protein was localized at the plasma membrane upon transient expression in onion epidermal cells (Engel *et al.*, 2005), and expression of *GEX1* was detected in sperm cells using a *ProGEX1:eGFP* reporter line (Engel *et al.*, 2005). The sequence of GEX1 is conserved among land plants, including monocots, eudicots, the moss Physcomitrella and the lycophyte Selaginella (Figure S1). Plasma membrane proteins that are expressed in gametic cells might play important roles in recognition and signaling during gametophyte development and/or during different steps of the fertilization process. To determine the role of GEX1 during these stages we used a reverse genetic approach, and characterized two T-DNA insertion lines: *gex1-1* and *gex1-3*. The T-DNA insertions were mapped to two different locations within the open reading frame (ORF) of *GEX1* (Figure 1a), potentially disrupting different protein domains.

Characterization of mutant phenotypes

To investigate if GEX1 had a role during the fertilization process, we first determined if the mutant lines had defects in seed development or seed set. We examined siliques of *gex1-1/+* and *gex1-3/+* plants 6–8 days after pollination (DAP). Both lines showed fewer wild type-like developed seeds than expected. Superficially, the *gex-1* and *gex1-3* phenotypes looked similar, but there were differences. The *gex1-1/+* plants showed approximately 25% reduced seed set ($22 \pm 5\%$ aborted seeds and $3 \pm 1\%$ undeveloped ovules; Figure 1c), whereas the *gex1-3/+* plants showed 49% reduced seed set ($12 \pm 2\%$ aborted seeds and $37 \pm 4\%$ undeveloped ovules; Figure 1d). We then examined pollen in open flowers. The *gex1-1*/+ plants had 100% mature tricellular pollen grains ($n = 1399$) (Figure S2a,f), but *gex1-3*/+ plants had 36% aborted pollen ($n = 1639$) (Figure S2b,g). In order to ascertain the genetic segregation of each mutant allele in self-pollinated progeny, we scored the ratio (resistant versus sensitive) of *gex1-1/+* and *gex1-3*/+ plants, using the linked basta or kanamycin (kan) resistance genes, respectively. For the *gex1-1* allele the segregation ratio was 1.82:1 (1135:622), whereas for the *gex1-3* allele the ratio was 1.65:1 (896:620).

These data suggested that each mutant allele partially affected the transmission of one or both gametophytes, and/or that the homozygote was embryo lethal. To distinguish among these hypotheses, we carried out reciprocal crosses of *gex1-1*/+ and *gex1-3*/+ plants with wild-type (WT) plants, and scored the transmission efficiency of each mutation, by selection on basta or kan, respectively. Table 1 shows that for *gex1-1* the transmission of the T-DNA insertion through the female gametophyte was complete (approximately 97%), but that transmission through the male gametophyte was slightly reduced (approximately 78%). When pollen from these plants was crossed with WT females, the resulting siliques (6–8 DAP) had nearly full seed set ($93 \pm 4\%$, $n = 520$), therefore indicating that the male gametophyte defect was not causing the 25% reduced seed set observed in the *gex1-1/+* plants. In contrast, heterozygous *gex1-3*/+ plants showed 41% transmission of the mutant allele through the female gametophyte, and 47% transmission of the mutant allele through the male gametophyte (Table 1). When *gex1-3*/+ plants were used as pollen donors on WT pistils, the siliques had nearly full seed set ($96 \pm 4\%$, $n = 312$), indicating, as in *gex1-1/+* plants, that the male gametophyte defect was not causing the reduced seed set observed in the *gex1-3*/+ plants.

When *gex1-3*/+ plants were used as females, however, the siliques had $62 \pm 8\%$ WT seeds, $36 \pm 7\%$ undeveloped ovules and $1 \pm 1\%$ aborted seeds ($n = 211$): this data is consistent with the reduced (41%) transmission of the *gex1-3* allele through the female gametophyte.

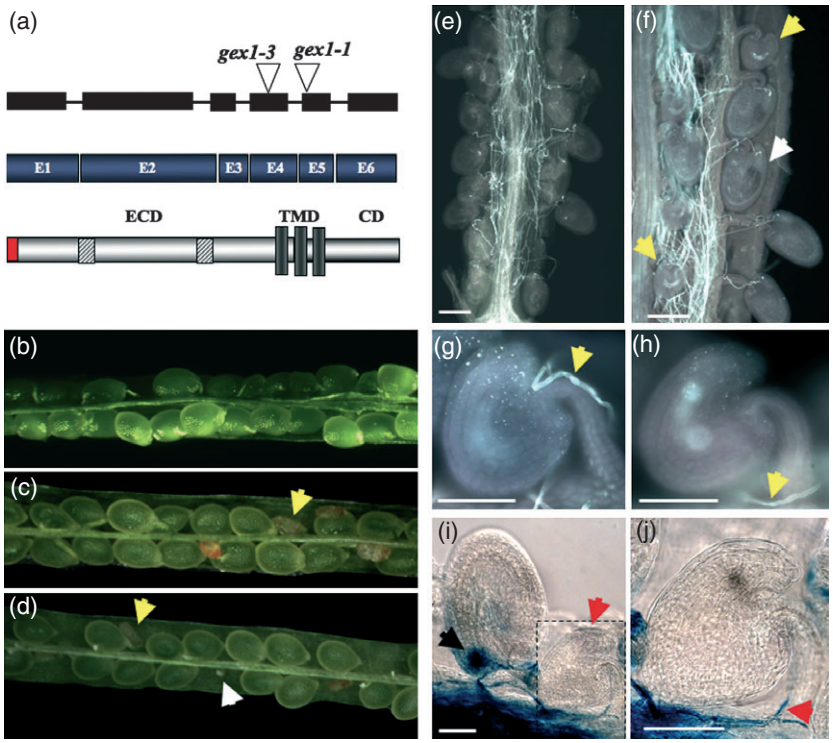

Figure 1. Phenotypic characterization of *gex1-1/+* and *gex1-3/+* plants.
(a) Top, diagram of genomic locus of *GEX1*, including exons (black boxes) and introns (lines), and locations of T-DNA insertions in *gex1-1* and *gex1-3*. Bottom, diagram of the predicted topology of GEX1, including a signal peptide in red and two coiled-coil motifs in grey.
(b) Silique (6–8 days after pollination, DAP) from a wild-type (WT) plant with full seed set.
(c) Silique (6–8 DAP) from a *gex1-1/+* plant showing aborted seeds (yellow arrow).
(d) Silique (6–8 DAP) from a *gex1-3/+* plant showing aborted seeds (yellow arrow) and undeveloped ovules (white arrow).
(e) Self-crossed pistil (1–2 DAP) of a *gex1-1/+* plant stained with DAB; all female gametophytes were reached by a pollen tube.
(f) Self-crossed pistil (1–2 DAP) of a *gex1-3/+* plant stained with DAB, showing female gametophytes that were fertilized after the arrival of a pollen tube (white arrow), and female gametophytes that were not reached by a pollen tube (yellow arrow).
(g and h) Unfertilized ovules showing pollen tube guidance defects of *gex1-3/+* plants (yellow arrow points pollen tube).
(i) Two ovules in a pistil (1–2 DAP) from a cross using *gex1-3/+* plant as female and the *ProLAT52:GUS* reporter line as a male. A fertilized ovule (black arrow points to a blue pollen tube at the micropyle), and a putative *gex1-3* ovule (red arrow) that failed to attract a pollen tube towards the micropyle.
(j) Close up of the putative *gex1-3* ovule in (i). CD, cytoplasmic domain; ECD, extracellular domain; TMD, transmembrane domain. Scale bars: e, f, 100 μm; g–j, 50 μm.

Table 1 Transmission Efficiency in *gex1-1/+* and *gex 1-3/+*

Parental Genotype (Female X Male)	Basta^R	Basta^S	Transmission Efficiency (TE) (%)	c2 Test
gex1-1/+ X WT	125	129	97	c2 = 0.063, *P* = 0.8018
WT X *gex1-1/+*	165	207	78	c2 = 4.742, *P* = 0.0294[a]
gex1-3/+ X WT	73	175	41	c2 = 41.952, *P* < 0.0001[a]
WT X *gex1-3/+*	74	156	47	c2 = 29.235, *P* < 0.0001[a]

Transmission efficiency = Resistant/ Sensitive × 100.
[a]TE is statistically significant from the 100% TE expected for a sporophytic mutation.

To determine if all embryo sacs were targeted by pollen tubes, we used decolorized aniline blue (DAB) to visualize pollen tubes in the pistils of *gex1-1/+* and *gex1-3/+* plants. We collected self-pollinated flowers at 1–2 DAP and stained the pistils with DAB. In *gex1-1/+* plants all ovules were reached by pollen tubes (Figure 1e), which is consistent with the full transmission of the *gex1-1* allele through the female. In the *gex1-3/+* plants 35 ± 6% (*n* = 615) of the ovules did not attract pollen tubes (Figure 1f–h), consistent with the reduced transmission of the *gex1-3* allele through the female. To confirm the female gametophyte defect we crossed pollen of the *ProLAT52:GUS* (Twell *et al.*, 1991)

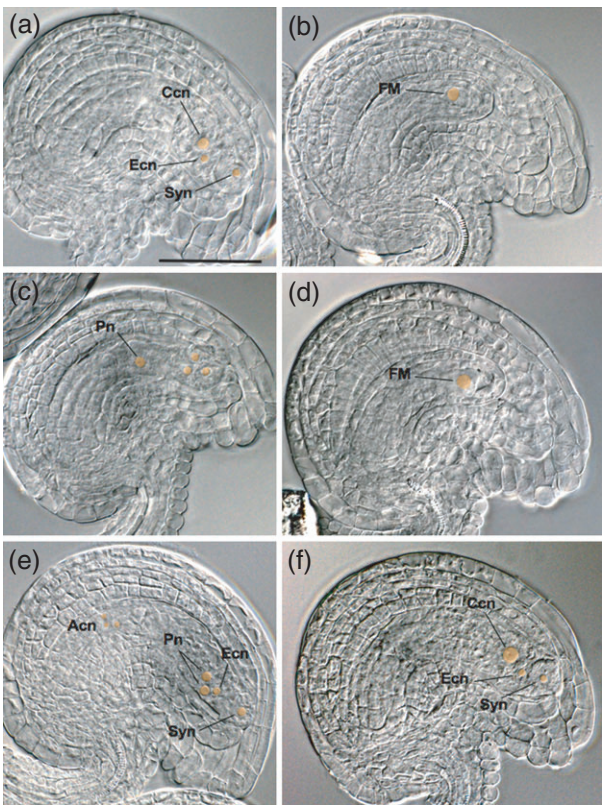

Figure 2. Female gametophyte development in *gex1-1/+* and *gex1-3/+* plants.
(a) Mature wild-type embryo sac after cellularization and differentiation (stage FG7) in emasculated pistils of *gex1-3/+* plants.
(b) Arrested embryo sac at FG1 in an ovule from the same pistil as in (a).
(c) Wild-type embryo sac at FG5 in pistils of *gex1-3/+* plants.
(d) Arrested embryo sac at FG1 in an ovule from same pistil as (c).
(e) Wild-type embryo sac at FG6 in pistils of *gex1-3/+* plants.
(f) Mature wild-type embryo sac after cellularization (stage FG7) in emasculated pistils of *gex1-1/+* plants. Ccn, central cell nucleus; Ecn, egg cell nucleus; FM, functional megaspore; Syn, synergid nucleus. Nuclei are false-colored.

reporter line with *gex1-3/+* plants: 37 ± 9% ovules (n = 209) were not reached by blue pollen tubes (Figure 1i,j) in siliques at 1–2 DAP. Because defects in female gametophyte development can cause a failure in pollen tube attraction (Marton and Dresselhaus, 2010), we decided to analyze mature female gametophytes of *gex1-3/+* plants to determine if there were abnormalities in cell polarity and/or in the position of the nuclei. Pistils of emasculated *gex1-3/+* plants had 63 ± 7% (n = 354) WT-like four-celled embryo sacs (FG7 stage) (Sundaresan and Alandete-Saez, 2010), but 36 ± 7% of the embryo sacs had arrested at the functional megaspore stage (FG1 stage) (Figure 2a,b, respectively). Younger pistils dissected from closed buds of *gex1-3/+* plants also carried both WT-like FG5 (eight nuclei) and FG6 (seven-celled and eight nuclei) stage embryo sacs, and embryo sacs that had arrested at the FG1 stage (Figure 2c,e,d, respectively). All mature female gametophytes in *gex1-1/+* plants showed correct cell polarity and nuclei position (Figure 2f). These results suggest that putative *gex1-3* female gametophytes were arrested at the FG1 stage, a phenotype that explains the reduced ability to attract pollen tubes observed in *gex1-3/+* plants.

gex1-1/gex1-1 and *gex1-3/gex1-3* affect early embryo development

The transmission and seed set results indicated that *gex1-1* and *gex1-3* mutant phenotypes were not fully penetrant, and therefore both the male and female gametophytes could transmit the mutant allele to the next generation. It should therefore be possible to obtain plants homozygous for these alleles. We selected seeds from self-pollinated *gex1-1/+* and *gex1-3/+* plants on basta or kan, respectively, and genotyped over 100 seedlings from each line. No homozygotes were identified for either mutant, suggesting that *gex1-1/gex1-1* and *gex1-3/gex1-3* are embryo lethal, which was consistent with the aborted seeds found in siliques of both mutant lines.

To determine the stage of embryogenesis that was compromised in the putative *gex1-1/gex1-1* and *gex1-3/gex1-3* embryos, we dissected and cleared seeds from siliques at different developmental stages and visualized them using differential interference contrast (DIC) imaging. Figure 3(a,b) shows normal embryo development at the triangular stage (5 DAP), and Figure 3(c,d) shows normal embryo development at the torpedo stage (6–7 DAP). Figure 3(e,f) shows abnormal embryo development in a putative *gex1-1/gex1-1* seed from the same silique as Figure 3(a), whereas Figure 3(g) and 3(h) show seeds with abnormal embryo development from the same silique as Figure 3(c). After the analysis of over 100 seeds showing abnormal embryo development, we observed that these embryos arrested between the two- and eight-celled embryo stage, and showed altered cell size in most cases, although abnormal arrested embryos at later developmental stages were also found. These seeds were slightly smaller than other seeds in the same silique, and showed endosperm nuclear divisions. We saw a similar arrested embryo development phenotype in a few seeds of self-pollinated *gex1-3/+* plants at 5 DAP (Figure 3i,j). To confirm that the endosperm development observed in the aborted seeds resulted from a product of fertilization, and not from autonomous endosperm development, we introgressed the reporter gene line *ProFAC1:GUS*, which is expressed in both embryo and endosperm upon fertilization (Xu *et al.*, 2005), into the *gex1-1/+* background. Figure 3(k) shows a WT developing seed that has GUS signal in the embryo and endosperm at 2 DAP. Figure 3(L) shows a putative *gex1-1/gex1-1* seed with GUS expression in the arrested embryo and developing endosperm at 2 DAP. We conclude that the putative *gex1-1/gex1-1* zygote/embryo arrested development soon after double fertilization, even though the endosperm continued developing (for at least 6–7 DAP) before the seed finally aborted.

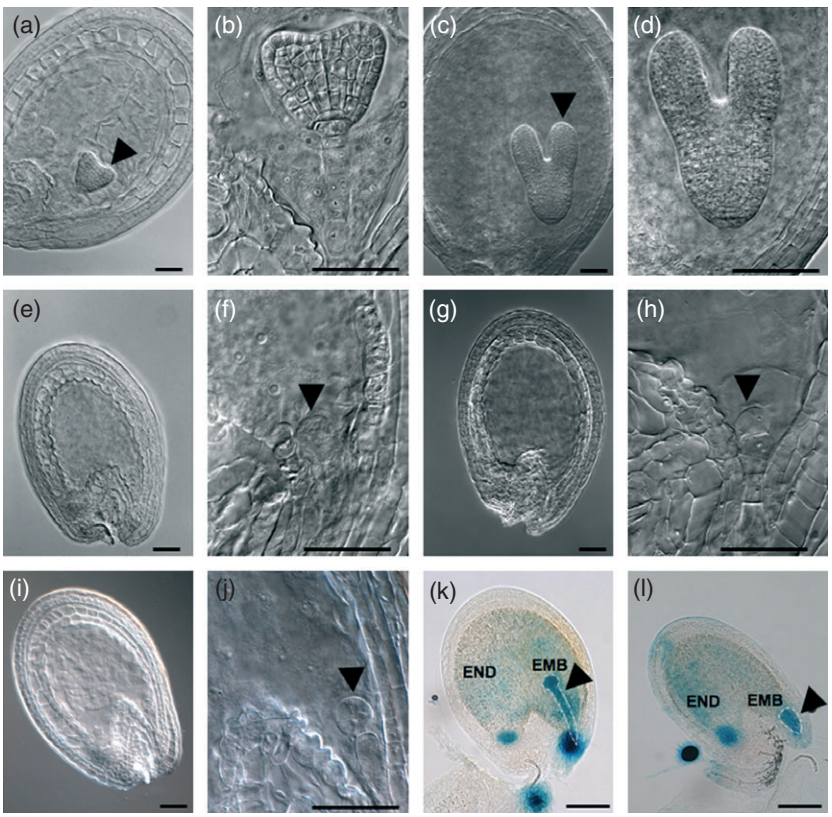

Figure 3. Embryo development in *gex1-1/+* and *gex1-3/+* plants.
(a) Wild-type (WT) embryo development at the heart stage (4 days after pollination, DAP) (arrow).
(b) Higher magnification of (a).
(c) WT embryo development at the torpedo stage (6–7 DAP) (arrow).
(d) Higher magnification of (c).
(e) Abnormal seed development (4 DAP) in *gex1-1/+* plants, with endosperm development and an arrested embryo.
(f) Higher magnification of (e) showing the arrested embryo (arrow).
(g) Abnormal seed development (6–7 DAP) in *gex1-1/+* plants, with endosperm development and an arrested embryo.
(h) Higher magnification of (g), showing the arrested four-celled embryo (arrow).
(i) Abnormal seed development (4 DAP) in *gex1-3/+* plants, showing endosperm development and an arrested embryo.
(j) Higher magnification of (e) showing the arrested two-celled embryo (arrow).
(k) WT seed (2 DAP) fertilized with pollen from the *ProFAC1:GUS* reporter line, showing GUS signal in the embryo (arrow) and the endosperm.
(l) Putative *gex1-1/gex1-1* seed (2 DAP) fertilized with pollen from the *ProFAC1:GUS* reporter line, showing GUS signal in the arrested embryo and the endosperm.
EMB, embryo; END, endosperm. Scale bar:50 μm.

We demonstrated that the phenotypes observed in the two mutant lines were caused by the corresponding T-DNA insertions by introducing a construct carrying the *GEX1* WT allele driven by its own promoter into the two mutant backgrounds. In each case, the phenotypes were fully complemented (Figure S3).

GEX1 is expressed in male and female gametophytes

The phenotypes identified in the insertion lines suggested a role for GEX1 in the female and male gametophyte, and in the embryo. To further evaluate *GEX1* expression, we generated the GUS reporter construct *ProGEX1:GUS*. A GUS signal was detected in the embryo sac before cellularization (a very weak signal at the FG1/FG2 stage and a more visible signal at the FG3/FG4/FG5 stage), in egg cells of mature female gametophytes and in zygotes/embryos of ovules at 1–2 DAP, but not in embryos older than 2 DAP (Figure 4a–f). This data is consistent with the gene expression map of Arabidopsis embryo development (http://www2.bri.nrc.ca/plantembryo) (Xiang *et al.*, 2011), which reported high expression levels of *GEX1* in the elongated zygote through the octant embryo stage. We also detected GUS in the vegetative cell, in a few pollen tubes upon pollen germination, and in root tips (Figure S4). RT-PCR using RNA from different tissues (Figure 4g) showed that *GEX1* was expressed strongly in pollen and weakly in unpollinated pistils. These additional expression data support the role of GEX1 during gametophyte development and early embryogenesis inferred from the *gex1-1* and *gex1-3* mutant lines.

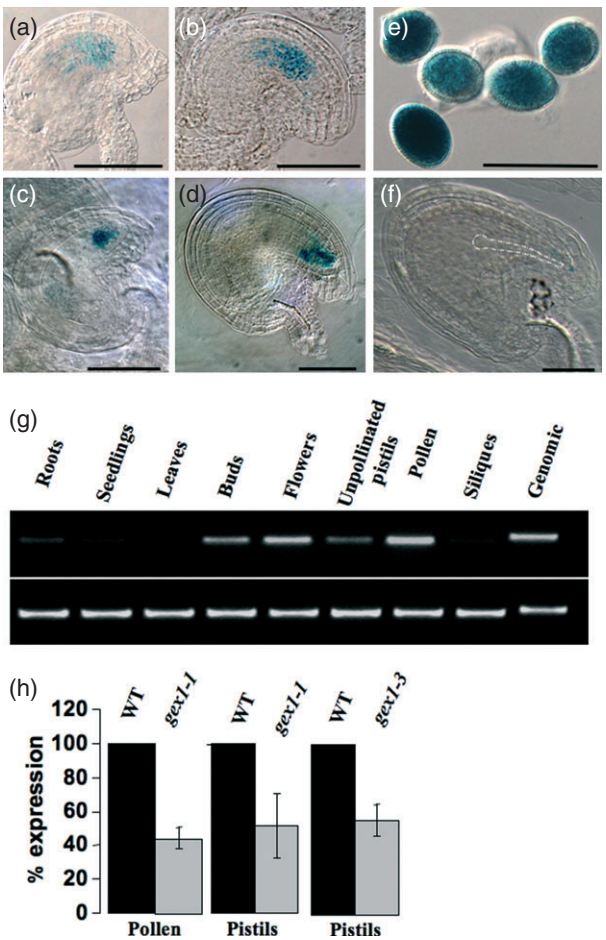

Figure 4. Expression in *ProGEX1:GUS* plants, and *GEX1* mRNA level in *gex1-1/+* and *gex1-3/+* plants.
(a, b) GUS expression in female gametophytes before the cellularization of *ProGEX1:GUS* plants.
(c) *GUS* signal in the egg cell of ovules dissected from unpollinated pistils.
(d) *GUS* signal in the zygote/embryo of ovules dissected from siliques 1 day after pollination (DAP).
(e) Mature pollen grains expressing GUS.
(f) Absence of GUS signal in seed at 2 DAP. The position of the embryo is shown with a dotted line.
(g) RT-PCR with eight different tissues of Arabidopsis, using *GEX1* (upper) and *ACTIN2* (lower) primers.
(h) *GEX1* mRNA levels measured using qRT-PCR in pollen and unpollinated pistils of WT and *gex1-1/+* plants; and in unpollinated pistils of WT and *gex1-3/+* plants. Wild-type levels were set to 100%. Scale bar:50 μm. Error bars represent standard deviation.

We performed quantitative real-time PCR (qRT-PCR) to analyze *GEX1* expression levels in the WT and in the mutant lines. We first extracted RNA from pollen and unpollinated pistils of *gex1-1/+* plants, and observed that *GEX1* mRNA levels were approximately 50% of WT levels in both (Figure 4h). Because the level of *GEX1* expression could be detected equally in pollen and unpollinated pistils, we used RNA from unpollinated pistils for further analysis, as they were easier to collect. The mRNA levels of *GEX1* in unpollinated pistils of *gex1-3/+* plants were approximately 50% of the levels found in WT plants (Figure 4h). In addition, we quantified *GEX1* expression with a third allele, *gex1-2*. The expression of *GEX1* in unpollinated pistils, anthers and leaves, and in pistils at 3 DAP, was higher in *gex1-2/+* plants than in the WT (Figure S5). The T-DNA insertion in *gex1-2/+* plants (FLAG line) contained a *CaMV35S* promoter immediately before the left border, and we therefore reasoned that higher expression of *GEX1* could be caused by such a promoter, as has happened for other T-DNA insertion lines (Williams *et al.*, 2005). We did not characterize *gex1-2* further.

gex1-1/+ plants express a truncated GEX1 protein *(gex1-1)* that localizes to the plasma membrane

We thought that the differences observed between the two alleles might be related to the position of the T-DNA insertion within the open reading frame (ORF) of *GEX1*, and we hypothesized that *gex1-1/+* plants might synthesize a truncated *GEX1* mRNA, up to the T-DNA insertion (Figure 5a), that might be translated and localized at the plasma membrane. To test this hypothesis we first determined *GEX1* mRNA levels using primers located upstream of the T-DNA insertion in *gex1-1/+* plants. qRT-PCR analysis showed that *GEX1* mRNA levels in unpollinated pistils were similar to WT levels (Figure 5b), suggesting that a partial *GEX1* mRNA was probably transcribed up to the location of the T-DNA insertion; when we used primers located downstream of the insertion we observed that *GEX1* mRNA expression was decreased to 50% of WT levels (Figures 4h and 5b). In addition, 3′ rapid amplification of cDNA ends (3′RACE) using RNA from unpollinated pistils of *gex1-1/+* plants detected two bands (Figure 5c), one corresponding to full-length *GEX1* mRNA and the second to a truncated *GEX1* mRNA up to the T-DNA insertion location, and including 51 nucleotides carrying a sequence that is complementary to the left border of the T-DNA, which would encode 16 amino acids before a stop codon.

Because this *GEX1-1* truncated mRNA was polyadenylated, we tested if the partial mRNA synthesized in *gex1-1/+* plants could be translated into a truncated protein lacking 129 amino acids (illustrated in Figure 5a), and if so, to determine its subcellular localization. We bombarded onion cells with a construct encoding the putative truncated version fused to GFP at the C terminus and under the control of the CaMV35S promoter (*Pro35S:gex1-1-eGFP*). Figure 5(d) shows that the WT version of GEX1 is located at the plasma membrane, as has already been shown by Engel *et al.* (2005). We also detected some perinuclear signal, most likely corresponding to the endoplasmic reticulum (Beraud-Dufour and Balch, 2002), and probably to the result of excess fusion protein in the exocytic pathway (Roselli *et al.*, 2004). Figure 5(f) shows that the truncated mRNA was translated into a protein that was also targeted to the plasma

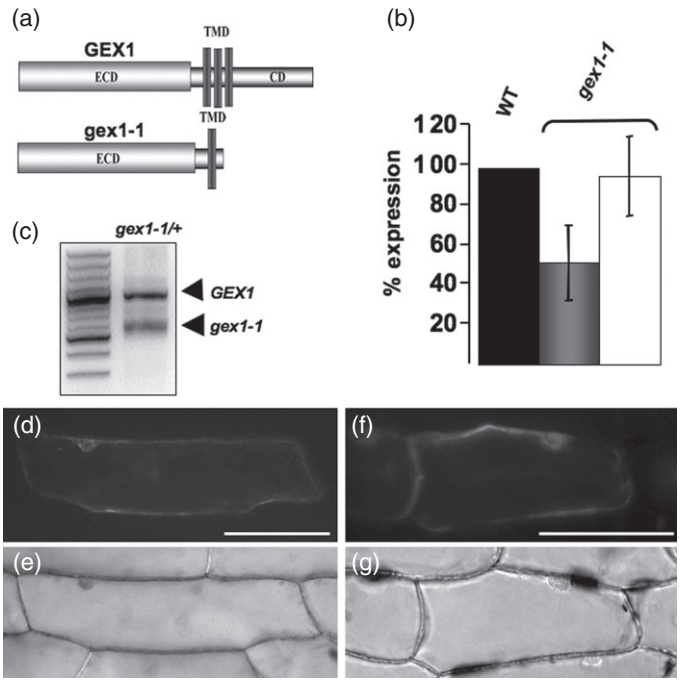

Figure 5. *GEX1* and *gex1-1* mRNAs are transcribed in *gex1-1/+* plants, and both proteins are targeted to the plasma membrane in onion cells.
(a) Predicted topology of full-length GEX1 (top) and truncated gex1-1 (bottom).
(b) qRT-PCR for *GEX1* expression using primers located downstream (dark grey) or upstream (white) of the T-DNA in unpollinated pistils of wild-type (WT) and *gex1-1/+* plants. Wild-type levels were set to 100% using both pairs of primers.
(c) Agarose gel showing the two bands detected in a 3′ RACE experiment, corresponding to the WT full-length *GEX1* mRNA (top) and the truncated *gex1-1* mRNA (bottom).
(d) GEX1-eGFP is located at the plasma membrane, as well as in the ER surrounding the nucleus, in onion epidermal cells.
(e) Differential interference contrast (DIC) image of (d).
(f) gex1-1-eGFP is located at the plasma membrane as well as in the endoplasmic reticulum surrounding the nucleus in onion epidermal cells.
(g) DIC image of (f).

membrane. The gex1-1-eGFP fusion protein also showed a weak perinuclear signal.

The truncated gex1-1 protein is equivalent to GEX1 during gametophyte development but not during early embryogenesis

Because *gex1-1/+* plants did not show pollen tube guidance defects or embryo sac arrest, nor the pollen abortion observed in *gex1-3/+* plants, we hypothesized that the truncated gex1-1 protein (Figure 5a) might be equivalent to GEX1 during male and female gametophyte development before double fertilization. If so, silencing the expression of the *gex1-1* truncated mRNA in the male and female gametophytes in *gex1-1/+* plants might phenocopy the defects observed in *gex1-3/+* plants. To test this hypothesis we generated transgenic lines silencing *GEX1* expression only in the gametophytes by using an antisense construct driven by the *GEX2* promoter that is not active in sporophytic tissues (Engel *et al.*, 2005), nor in the zygote or embryo, according to the gene expression map of Arabidopsis embryo development (http://www2.bri.nrc.ca/plant-embryo; Xiang *et al.*, 2011), but is expressed both in the sperm (Engel *et al.*, 2005) and in the egg (Alandete-Saez *et al.*, 2008).

We identified 15 primary (T_1) transformants that showed differing degrees of reduced seed set, i.e. approximately 25–65% undeveloped ovules. Because plants showing more than 50% reduced seed set might carry multiple T-DNA insertions, we grew T_2 and T_3 segregating plants from four different AS families (AS5, AS55, AS11 and AS35) that had shown up to 50% reduced seed set (Figure S6), and then quantified *GEX1* mRNA levels in unpollinated pistils and stamens using qRT-PCR. A silique of one such antisense plant (AS5) at 6–8 DAP, with 50 ± 5% undeveloped ovules (Table 2), is shown in Figure 6(b). Figure 6(c,d) shows that *GEX1* expression was reduced to approximately 50% in all four lines in both tissues. We collected self-pollinated pistils from these four lines at 1–2 DAP and stained them with DAB. In all four lines undeveloped ovules did not attract pollen tubes, a phenotype similar to that observed in *gex1-3/+* plants (Figure 6e). We confirmed that pollen tube guidance defects were caused by the female gametophyte by using a

Table 2 Seed set in mature siliques (6–8 DAP) of Col, *gex1-1/+*, *AS5/-* and *gex1-1/+; AS5/-*

Genotype	Developed (%)	Aborted (%)	Undev. (%)	Total (n)	c2 Test
Col	1026 (98)	20 (2)	0	1046	N/A
gex1-1/+	769 (75)	225 (22)	31 (3)	1025	c2 = 2840.2, $P < 0.0001$[a]
AS5/-	929 (49)	19 (1)	947 (50)	1895	c2 = 23126.4, $P < 0.0001$[a]
gex1-1/+;AS5/-	470 (43)	109 (10)	514 (47)	1094	c2 = 23126.4, $P < 0.0001$[a]

[a]Numbers of aborted and undeveloped seeds are statistically significant from that in Col plants.

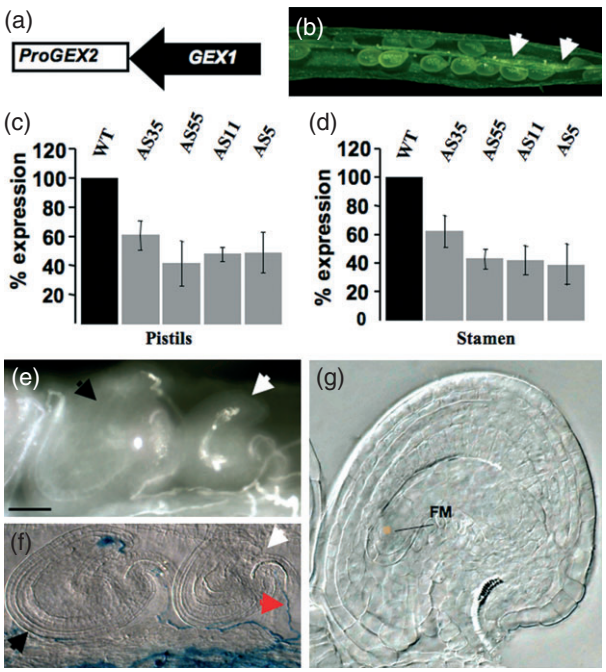

Figure 6. Phenotypic characterization of *GEX1* AS transgenic lines.
(a) Diagram of the AS construct used to downregulate *GEX1* using the *GEX2* promoter.
(b) Silique (6–8 days after pollination, DAP) from a *GEX1* AS5 plant showing undeveloped ovules (arrows).
(c) QPCR for *GEX1* expression in unpollinated pistils of wild-type (WT) and four independent transgenic lines for the *GEX1 AS* construct. Wild-type levels were set to 100%.
(d) QPCR for *GEX1* expression in stamens of WT and four independent transgenic lines for the *GEX1 AS* construct. Wild-type levels were set to 100%.
(e) Self-crossed pistil (1–2 DAP) of a *GEX1 AS5* plant stained with DAB, showing a fertilized ovule after the arrival of a pollen tube to the micropyle (black arrow) and an ovule that failed to attract a pollen tube (white arrow).
(f) Pistil (1–2 DAP) from a cross using the *GEX1 AS5* line as female and the *ProLAT52*: *GUS* reporter line as male. The black arrow points a fertilized ovule (blue pollen tube at the micropyle) and an ovule (white arrow) that failed to attract the pollen tube (red arrow) towards the micropyle.
(g) Ovule from an emasculated pistil of the *GEX1 AS5* line showing an arrested female gametophyte at the FG1 stage. Nucleus is false-colored yellow. FM, functional megaspore.

ProLAT52:GUS reporter line as the pollen donor (Figure 6f) and the antisense line AS5 as the female. We further analyzed line AS5 and observed 36% pollen abortion (Figure S2c, n = 1582; Table S1) and 45% embryo sacs arrested at the FG1 stage (Figure 6g, n = 207), similar to the phenotypes observed in the *gex1-3/+* plants. These data indicated that the antisense construct driven by the *GEX2* promoter was active in pollen and during the early stages of female gametophyte development. We did not observe seed abortion in the antisense lines, probably because the *GEX2* promoter is not active in the embryo/zygote.

To further test our hypothesis, we crossed a *gex1-1/+* plant as the female with the *GEX1* AS5 line as the male. We then genotyped the F_1 seedlings and obtained WT, *gex1-1*/+, *GEX1* AS/– and *GEX1* AS/–;*gex1-1*/+ plants. Table 2 shows that WT plants had full seed set, *gex1-1*/+ plants had 22 ± 5% aborted seeds, *GEX1* AS/– plants had 50 ± 5% undeveloped ovules and *GEX1* AS/–;*gex1-1*/+ plants had 47 ± 6% undeveloped ovules and 10 ± 2% aborted seeds. These aborted seeds were putative *gex1-1/gex1-1* seeds, either from the fertilization of embryo sacs (*gex1-1*) with sperm cells carrying the *gex1-1* allele, but lacking the *GEX1* AS construct (*gex1-1*;–), or from the small proportion of pollen grains carrying both the *gex1-1* allele and the AS construct (*gex1-1*;*GEX1* AS) that did not abort and were able to reach *gex1-1* embryo sacs. Undeveloped ovules in the self-pollinated pistils of *GEX1* AS/–;*gex1-1*/+ plants at 1–2 DAP failed to attract pollen tubes, as did the *GEX1* AS5 line, and had 36% pollen abortion (Figure S2d; Table S1). We did not recover a *gex1-1/gex1-3* plant from crosses using *gex1-1/+* as a female and *gex1-3/+* as a male.

These results indicated that silencing *GEX1* expression using an antisense construct phenocopied the *gex1-3* mutation, and also that the truncated gex1-1 protein expressed in *gex1-1*/+ plants was equivalent to the WT GEX1 protein during the development of male and female gametophytes, even though the gex1-1 truncated protein lacks the 129 amino acid cytoplasmic domain). However, putative *gex1-1*/*gex1-1* embryos were arrested at an early stage because of the absence of the GEX1 cytoplasmic domain.

GEX1 forms homodimers interacting through the cytoplasmic domain

Because it is common that proteins located at membranes form dimers to carry out their functions, we hypothesized that GEX1 might form homodimers in the plasma membrane. To test this hypothesis, we used the BiFC assay (Citovsky *et al.*, 2006) and transient expression in onion epidermal cells. The full length (FL) amino acid sequence of GEX1 was fused to either the N terminus or C terminus of yellow fluorescent protein (YFP) as a C-terminal fusion. Figure 7(a) shows that WT GEX1 (FL) interacts with itself in the plasma membrane, indicating that GEX1 forms homodimers when expressed in the same cell. We next tested if the truncated gex1-1 protein could dimerize with either the WT GEX1 or another gex1-1 protein (Figure 7b,c). In neither case was a fluorescent signal detected in the plasma membrane, even though the bombardment assay worked efficiently (many transformed cells expressed the *Pro35S:GUS* construct that was co-bombarded with the spYFP vector). That the gex1-1 protein could not interact with GEX1 or another gex1-1 protein suggested that the interaction to form homodimers occurred through the transmembrane domains, through the cytoplasmic domains or through both. We therefore made a new construct encoding the signal peptide at the N terminus, followed by the three transmembrane domains and the cytoplasmic domain, fused to either N-YFP or C-YFP. Figure 7(d,e) shows that this protein could interact with both full-length WT GEX1 and with itself, confirming that GEX1

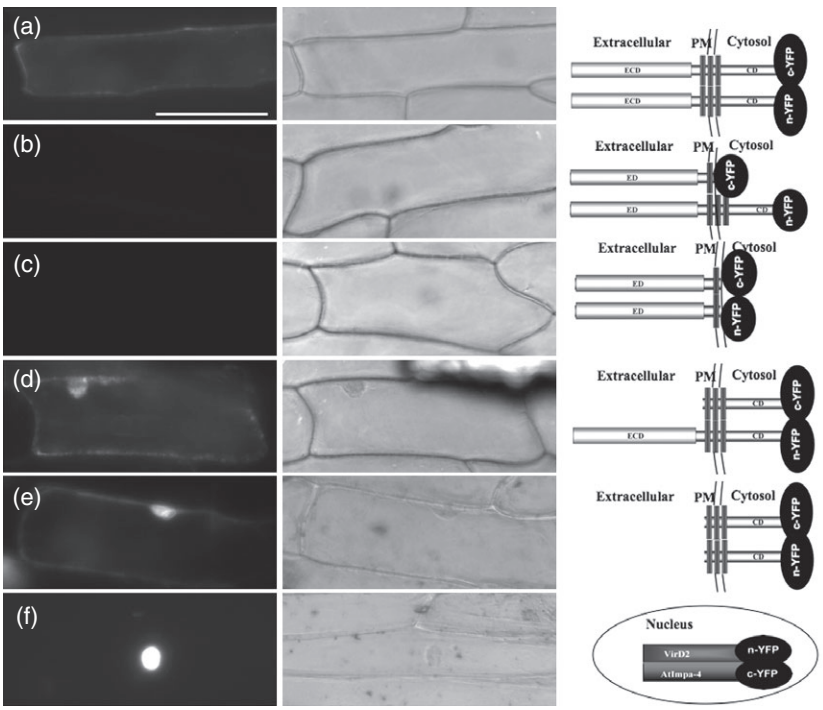

Figure 7. GEX1 forms homodimers in the plasma membrane through its cytoplasmic domain (CD). For each panel, the bimolecular fluorescence complementation assay is shown on the left, the differential interference contrast image is shown in the center, and the diagram and topology of the interacting proteins is shown on the right.
(a) GEX1-nCFP and GEX1-cYFP (FL) interact in the plasma membrane.
(b) GEX1 (FL) does not interact with *gex1-1* in the plasma membrane.
(c) *gex1-1* does not interact with *gex1-1* in the plasma membrane.
(d) GEX1 (FL) interacts with gex1-CD in the plasma membrane, as well as in the endoplasmic reticulum surrounding the nucleus.
(e) gex1-CD interacts with gex1-CD in the plasma membrane, as well as in the ER surrounding the nucleus.
(f) nEFP-VirD2 interacts with AtImpa-4cYFP in the nucleus. CD, cytoplasmic domain; ECD, extracellular domain; PM, plasma membrane; TMD, transmembrane domain. Scale bars:100 μm.

forms homodimers through its transmembrane and cytoplasmic domains. We observed perinuclear fluorescence with all GEX1 protein versions that showed positive interactions in the BiFC assay, which was consistent with the subcellular localization of both the full-length GEX1 and gex1-1 truncated proteins. In addition, we tested whether the extracellular domain (ECD) of GEX1 could interact with itself in the opposite orientation, because that would suggest that GEX1 could form homodimers when in the plasma membrane of two different cells (e.g. egg and sperm cells, before or during the fusion of their membranes). We used the predicted ECD of GEX1, without the predicted signal peptide, fused to either the N terminus or the C terminus of YFP as C-terminal or N-terminal fusions, but did not detect any interaction between the extracellular domains.

DISCUSSION

GEX1 was previously shown to be expressed in sperm cells (Engel *et al.*, 2005; Borges *et al.*, 2008; Slotkin *et al.*, 2009) as well as in other sporophytic tissues using a GFP reporter construct (Engel *et al.*, 2005). Here we used a more sensitive GUS reporter to show that *GEX1* is also expressed during early embryo sac development and in the egg cell, but also during the first divisions of the zygote/embryo (Figure 4a–d). We did not detect GUS signal in the sperm cells, possibly because expression in the vegetative cell masked the sperm cell signal, or because the promoter used for the GUS reporter construct (same promoter used in the complementation constructs) was slightly smaller than that used for the *ProGEX1-eGFP* construct (upstream of the ATG at 1606 versus 1808 bp). Using a reverse genetic approach we showed that GEX1 is a versatile protein of dual function during gametophyte development and early embryogenesis. Our genetic approach was unable to uncover a role for GEX1 in sperm cells during fertilization, because of its earlier role during pollen development, although that possibility cannot be ruled out. The *gex1-3* T-DNA insertion, located in the fourth exon (Figure 1a), disrupts *GEX1* mRNA transcription before the sequence encoding its transmembrane domain and, based on qRT-PCR data, is a null allele. *gex1-3/+* plants showed defects in pollen (Figure S2), embryo sac (Figure 2) and early embryo development (Figure 3). Although it is well documented that the embryo transcriptome shares a high number of expressed genes with both

the male and female gametophytes (Haecker *et al.*, 2004; Berg *et al.*, 2005; Jenik *et al.*, 2007; Spencer *et al.*, 2007; Bayer *et al.*, 2009; Wuest *et al.*, 2010), there is limited information linking the expression pattern of a gametophytically expressed gene with a role during early embryogenesis (Bayer *et al.*, 2009). The embryo sacs carrying the *gex1-3* allele showed arrested development at the FG1 stage (Figure 2), and did not attract pollen tubes (Figure 1f); most likely because of the absence of attractant molecules required for micropylar pollen tube guidance, which are only secreted when female gametophytes are fully mature (Higashiyama, 2002; Palanivelu and Preuss, 2006; Dresselhaus and Marton, 2009). In contrast to mutations in other sperm-expressed genes (von Besser *et al.*, 2006; Mori *et al.*, 2006; Nowack *et al.*, 2006; Chen *et al.*, 2008; Ron *et al.*, 2010), *gex1-3* pollen grains did not show defects in pollen tube guidance, pollen tube reception or fertilization. Instead, *gex1-3* pollen grains aborted, suggesting that GEX1 is essential for correct pollen maturation, as is the case for other pollen-expressed genes (Chen and McCormick, 1996; Boavida *et al.*, 2009). *GEX1* is an essential gene in Arabidopsis because both *gex1-3/gex1-3* and *gex1-1/gex1-1* embryos arrested at an early stage. Even though *GEX1* is expressed in the male and female gametic cells, reciprocal crosses indicated that the embryo arrest phenotype was caused by a recessive mutation and was not maternally or paternally imprinted, as is the case for the *glauce* and *short suspensor* mutants, respectively (Ngo *et al.*, 2007; Bayer *et al.*, 2009).

Half of the gametophytes produced by *gex1-1/+* plants expressed the WT full-length *GEX1* mRNA, and the other half expressed truncated *gex1-1* mRNA. We showed that both transcripts (*GEX1* and *gex1-1*) could be translated into proteins that were targeted to the plasma membrane (Figure 5c). *gex1-1/+* plants did not have the gametophytic defects observed in *gex1-3/+* plants. Three lines of evidence support the hypothesis that the predicted extracellular domain of GEX1, when inserted at the plasma membrane, is sufficient and necessary for the development of both male and female gametophytes: (i) *gex1-1*/+ plants had aborted seeds because of embryo arrest, and not the gametophytic defects observed in *gex1-3/+* plants (Figure 3); (ii) transgenic lines carrying a *GEX1* antisense construct phenocopied the gametophytic defects observed in *gex1-3* plants, consistent with the downregulation of *GEX1* transcripts (Figure 6); and (iii) the introduction of the antisense construct in the *gex1-1* background phenocopied the gametophytic defects caused by the *gex1-3* mutation, which is consistent with the downregulation of *GEX1* and *gex1-1* transcripts (Table 2). GEX1 formed homodimers in the plasma membrane, and its predicted cytoplasmic domain (CD) was sufficient and necessary for GEX1 interaction and function during early embryogenesis (Figure 7). However, the fully functional gametophytes found in *gex1-1/+* plants indicate that the role of the predicted extracellular domain (ECD) of GEX1 during gametophyte development does not require GEX1 dimerization. Although we did not obtain evidence that the extracellular domain of GEX1 can interact with itself when in opposite orientations, these BiFC experiments cannot rule out the possibility that GEX1 can form homodimers when in the plasma membrane of two different cells, because the interaction might require specific protein folding in the plasma membrane. Coiled-coil domains are found in diverse proteins and mediate protein–protein interactions (Suzuki *et al.*, 2008). The presence of two coiled-coil domains in the predicted extracellular domain of GEX1 (Figure 1a) support the idea that the extracellular domain might interact with itself or with other proteins.

The strong GUS signal observed in embryos at 1–2 DAP (Figure 4d) and microarray expression data reported in the gene expression map of Arabidopsis embryo development (http://www2.bri.nrc.ca/plantembryo; Xiang *et al.*, 2011) support the speculation that *de novo* transcription/translation of *GEX1* occurs immediately after karyogamy, instead of by protein diffusion in the plasma membrane after fusion of the gametic cells, as is true for other plasma membrane proteins (Hink *et al.*, 2008). The *de novo* translated GEX1 would then form dimers in the plasma membrane through its cytoplasmic domain, and we hypothesize that this dimerization would be required for an upstream step in a signaling cascade involved in the establishment and development of the early embryo. It would be interesting to investigate if the homeobox transcription factors WOX2 and WOX8, which act as complementary cell fate regulators in the apical and basal lineages during early embryogenesis (Breuninger *et al.*, 2008), and are co-expressed in both the egg cell and zygote (Haecker *et al.*, 2004), might be downstream players in this signaling cascade.

EXPERIMENTAL PROCEDURES

Primers for cloning and genotyping are listed in Table S2.

Plant materials and growth conditions

Arabidopsis plants (Columbia, Col-0) were grown in a glasshouse as described by Alandete-Saez *et al.* (2008). Seeds were plated on MS medium containing 30 μg ml^{-1} BASTA (*gex1-1/+* and *GEX1* AS plants) or 50 μg ml^{-1} kanamycin (*gex1-2/+* and *gex1-3/+* plants), and the resulting seedlings were scored for resistance 2 weeks later.

T-DNA line and sequence analyses

The left borders of the T-DNA insertions CS817262 (*gex1-1*), FLAG_48E09 (*gex1-2*) and FLAG_081B03 (*gex1-3*) were amplified by PCR and sequenced to verify the insertion sites. The structure and subcellular localization of GEX1 were predicted as described in Alandete-Saez *et al.* (2008).

Cloning and generation of transgenic lines

All constructs were subcloned into pENTR 3C or pENTR/D-TOPO vectors (Invitrogen, http://www.invitrogen.com), confirmed by sequencing and transferred to destination vectors by an LR reaction using LR Clonase II (Invitrogen). The full-length *GEX1* open reading

frame was amplified from *Arabidopsis* pollen cDNA using primers MA7/MA8, and was cloned in an antisense orientation into pB7WG2*-*ProGEX2*, as in (Alandete-Saez *et al.*, 2008). The *GEX1* promoter (1.6 kb) was amplified using primers MA9/MA10 and cloned into pBGWFS7 (Karimi *et al.*, 2002) to generate the reporter line *ProGEX1:eGFP-GUS*. For the complementation construct a 3.8-kb fragment of *GEX1*, including the promoter and 3′ untranslated region (UTR), was amplified from genomic DNA using primers MA11/MA12 and cloned into a modified pK2GW7 (for *gex1-1* complementation) or pH2GW7 (for *gex1-3* complementation) vectors (Karimi *et al.*, 2002), from which the *CaMV35S* promoter had been removed to generate *ProGEX1:GEX1* complementation constructs.

Phenotypic analysis and complementation of *gex1-1/+* and *gex1-3/+* plants

Phenotypic analyses of seed set and reciprocal crosses were performed as described in Alandete-Saez *et al.* (2008). Decolorized aniline blue (DAB) staining was performed as described in Mori *et al.* (2006). GUS expression analyses were performed as described in Johnson *et al.* (2004), and seed clearings were performed as described in Pagnussat *et al.* (2005). To complement the *gex1-1* mutant, the *ProGEX1:GEX1* complementation construct was introduced into Col-0 plants using *Agrobacterium* transformation (Clough and Bent, 1998), then pollen from transgenic T_1 lines was used for crosses with *gex1-1/+*. Seedlings from these crosses were selected on kanamycin and were genotyped for the presence of the *gex1-1* T-DNA. Five independent *ProGEX1:GEX1*; *gex1-1/+* F_1 plants were taken to the next generation. Seeds were selected on kanamycin and homozygous *gex1-1/gex1-1/GEX1* complementation plants were identified by PCR. To complement the *gex1-3* mutant, the *ProGEX1:GEX1* complementation construct was introduced into *gex1-3/+* plants. T_1 lines were selected on hygromycin and genotyped for the presence of the *gex1-3* T-DNA. Positive T_1 plants were allowed to self and T_2 seedlings were selected on both hygromycin and kanamycin. Homozygous *gex1-3/gex1-3/GEX1* complementation plants were identified by PCR.

RT-PCR and real-time PCR

RNA was extracted from different tissues using an RNeasy plant miniprep kit (Qiagen, http://www.qiagen.com) with on-column DNAse treatment. cDNA was synthesized using 20-mer oligod(T) and Superscript™ III Reverse Transcriptase (Invitrogen). RT-PCR was performed using 1 μg of total RNA. Real-time PCR primer pairs were designed using PRIMER EXPRESS (Applied Biosystems, http://www.appliedbiosystems.com). *IPP2* (*At3g02780*) was used for normalization, as described in Alandete-Saez *et al.* (2008). Amplification was performed with a MyIQ Real-Time PCR Detection System (Bio-Rad, http://www.bio-rad.com), with 1.5 μg and 500 ng of total RNA from unpollinated pistils and anthers, respectively.

Generation of clones for subcellular localization and BiFC assays

For subcellular localization, the open reading frames of *GEX1* or of *gex1-1*, without the stop codons, were amplified from cDNA prepared from unpollinated pistils and cloned into pB7FWG2 (Karimi *et al.*, 2002) to generate *Pro35S:GEX1-eGFP* and *Pro35S:GEX1-1-eGFP*. To generate constructs for BiFC, all fragments were amplified from cDNA prepared from unpollinated pistils and cloned into either pSAT4-nYFP-N1 or pSAT4-cYFP-N1 (Citovsky *et al.*, 2006). The full-length *GEX1* sequence was amplified using the primers MA30/MA31 and the truncated *gex1-1* was amplified using primers MA30/MA32. The predicted GEX1 ECD without the signal peptide was amplified using primers MA33/MA34 or MA35/MA36. The cytoplasmic domain construct was cloned by overlapping PCR. The signal peptide (36 amino acids) was amplified with the primers MA37/MA38 and the sequence encoding the transmembrane and cytoplasmic domains was amplified using the primers MA39/MA40. The amplicons were gel-purified and 0.5 μl of each fragment was used as template for a second PCR reaction (50 μl) with the primers MA37/MA40 to amplify the full-length overlapping sequence. These constructs were used for particle bombardment in onion epidermal cells. Microcarriers were prepared with 5 μg of plasmid for subcellular localization or 2 μg of each construct (i.e. nYFP-fused and cYFP-fused) for BiFC, including 500 ng of the *Pro35S:GUS* construct, mixed and coated onto 1.0-mm gold particles (Seashell Technology, http://www.seashelltech.com) according to the manufacturer's protocol. The particles were bombarded into peeled epidermal layers of onion using a PDS-100/He particle delivery system (Bio-Rad). The epidermal layers were incubated in the dark at 22°C and observed 16–20 h after bombardment using a Zeiss Axiovert microscope (Zeiss, http://www.zeiss.com). As negative controls, each protein-nYFP and protein-cYFP fusion construct was bombarded with the complementary half-YFP vector; both empty vectors were also tested.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Amino acid sequence alignment of GEX1.

Figure S2. Pollen phenotypes of *gex1-1/+*; *gex1-3/+*, *GEX1 AS/–* and *gex1-1/+;GEX1 AS/–* plants.

Figure S3. Complementation of *gex1-1* and *gex1-3* phenotypes.

Figure S4. *GEX1* expression in plants carrying the *ProGEX1:GUS* construct.

Figure S5. Quantification of *GEX1* mRNA levels by qRT-PCR in plants carrying the *gex1-2* T-DNA insertion.

Figure S6. Seed formation in mature siliques of four independent *GEX1 AS* lines.

Table S1. Pollen in open flowers of Col, *gex1-1/+*, *AS5/–* and *gex1-1/+*; *AS5/–* plants.

Table S2. Primers used in this study.

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